DOCTOR OF PHILOSOPHY

Cooperation between peroxisome proliferator activated receptor alpha and delta in regulation of body weight and hepatic steatosis in mice

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COOPERATION BETWEEN PEROXISOME PROLIFERATOR ACTIVATED RECEPTOR ALPHA AND DELTA IN REGULATION OF BODY WEIGHT AND HEPATIC STEATOSIS IN MICE

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ABBREVIATIONS

^{1}H-MRS: proton magnetic resonance spectroscopy
ABCA1: ATP-binding cassette transporter
Abcc3: ATP-binding cassette, sub-family C, member 3
Acox1: Acyl-Coenzyme A Oxidase 1
Acs: acyl-CoA synthetase
ADRP: Adipose Differentation-Related Protein
AFLD: alcoholic fatty liver disease
AhR:ArylHydrocarbon Receptor
ALT: alanine transaminase
ANGPTL4: Angiopoietin-Related Protein 4
Apo B: apolipoprotein B
ApoA5: Apolipoprotein A5
Apoc3: Apolipoprotein C3
AST: aspartate transaminase
BAT: Brown Adipose Tissue
BMI: body mass index
ChREBP: carbohydrate response element-binding protein
CPT1:CarnitinePalmitoyltransferase I
DMSO: Dimethyl sulfoxide
DR-1: direct repeat with a single nucleotide spacer
EDTA: ethylenediaminetetraacetate
FA: fatty acids
FABP: fatty acid binding protein
FAO: fatty acid oxidation
FAS: Fatty Acid Synthase
FAT: fatty acid translocase
FATP: fatty acid transport protein
FDR: false discovery rate
FFA: free fatty acids
HCC: hepatocellular carcinoma
HDAC: histone deacetylation activity
HDL: High Density Lipoprotein
HFD: High Fat Diet
HMG-CoAs: 3-hydroxy-3-methylglutaryl-CoA synthase
HSL: hormone sensitive lipase
I3C: Indole 3 Carbinol
Igf1: Insulin growth factor 1
IHTG: intrahepatic triglyceride
IL-6: interleukin 6
Insig-1: Insulin-Induced Gene 1
KO: Knockout
LC-MS: Liquid chromatography–mass spectrometry
LDLR: low density lipoprotein receptor
LPL: lipoprotein lipase
LXR: liver X receptor
Ly6d: Lymphocyte antigen 6 complex, locus D
MCD: methionine and choline-deficient diet
MTP: microsomal transfer protein
NAFLD:Non-Alcoholic Fatty Liver Disease
NASH: non-alcoholic steatohepatitis
NEFA: Non-Esterified Fatty Acids

OLETF: Otsuka Long Evans Tokushima Fatty rats
ORO: oil red oil
PBS: phosphate buffered saline
PC: phosphatidyl choline
PDK4: Pyruvate Dehydrogenase Kinase Isozyme 4
PFA: Paraformaldehyde
POPC: 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine
PPAR: Peroxisome Proliferator Activator Receptor
PPRE: Peroxisome Proliferator Response Element
RT-PCR: real time polymerase chain reaction
RXR: retinoid X receptor
S3-12: Plasma membrane associated protein
Slc19a1: Solute carrier family 19, member 1
Slc25a10: Solute carrier family 25, member 10
SREBP-1:Sterol Regulatory Element-Binding Protein
Taf1d: TATA box binding protein (Tbp)-associated factor, RNA polymerase I, D
TG: triacylglycerols, triglyceride
TZD: thiazolidinedione
UCP1: uncoupling protein 1
UCP2: uncoupling protein 2
UCP3: uncoupling protein 3
VAI: visceral adipose index
VAT: visceral adipose tissue
VLDL: very low density lipoprotein
WAT: White Adipose Tissue

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DECLARATION

I declare that this thesis is based upon results obtained from investigations performed by myself and that the entire thesis is my own composition. Any work other than my own has been clearly acknowledged in the text with reference to the relevant investigators and contributors. This thesis has not been previously presented, in whole or in part, for the award of any higher degree.

Signature.....

Date.....27/09/2012.....

Wojciech Grzegorz Garbacz

I confirm that Wojciech Grzegorz Garbacz has spent the equivalent of at least nine terms in research at the Medical Research Institute, Ninewells Hospital and Medical School, University of Dundee, Dundee, and that he has fulfilled the condition of the University of Dundee thereby qualifying him to submit this thesis in application for the degree of Doctor of Philosophy.

Signature.....

Date.....27/09/2012.

Professor Colin Palmer

ABSTRACT

Peroxisome proliferator-activated receptor alpha (PPARα) and delta (PPARδ) belong to the nuclear receptor superfamily. PPARα is a target of lipid-lowering drugs and PPARδ promotes fatty acid utilization and is a promising anti-diabetic drug target. However, evidence is growing that PPARδ-agonism can stimulate fat accumulation in liver, which may aggravate the toxic situation in diabetics.

The aim of the study was to characterise the hepatic transcriptional and lipid response of humanized mouse models to PPARδ-agonists.

In our studies of mice conditionally-expressing human PPARδ (hPPARδ), or the dominant-negative derivative of hPPARδ (hPPARδΔAF2) or wild-type animals, we demonstrated that GW501516, a potent PPARδ activator, promoted up-regulation of the genes involved in lipid turnover, stimulated significant weight loss and promoted hepatic steatosis in these mouse models. There was time-dependent accumulation of hepatic triglycerides observed in wild-type and in conditionally-expressing hPPARδ mice fed a diet containing PPARδ synthetic ligand. This was not seen in animals conditionally-expressing hPPARδΔAF2, neither in PPARα-KO or PPARδ-KO animals. Concurrently, activation of PPARδ in humanised animals caused significant depletion, as compared with controls, of adipose tissue deposits when fed normal or high fat diet. This effect was completely absent in PPARα-KO or PPARδ-KO mice, fed diet containing GW501516. Genome-wide transcriptional profiling of GW501516 effects in the livers of these different mouse strains was performed. In PPARα-KO mice fed PPARδ-agonist, some

direct PPARδ target genes were still up-regulated, demonstrating that they are not sufficient for the observed phenotype. In addition the blood HDL-raising effects of GW501516 were preserved in the PPARα-KO mice.

This suggests a novel finding that both PPARδ and PPARα receptors are essential for GW501516-driven weight loss and hepatic steatosis, with PPARα working downstream of PPARδ.

Chapter 1 Introduction to fatty liver disease

1.1 Liver lipid metabolism

The liver is an organ that performs a diverse array of biochemical functions necessary for whole-body metabolic homeostasis. One of them is turnover of lipids. Fatty acids (FA) are the most frequently stored and circulating forms of energy, and triacylglycerols (TG) are the non-toxic form of fatty acids. Fatty acids/triacylglycerols may appear in liver from four sources. *De novo* fatty acids synthesis (*de novo* lipogenesis), cytoplasmic triacylglycerol stores, fatty acids derived from triacylglycerols of lipoprotein remnants directly taken up by the liver, and plasma free fatty acids released by adipose tissue [1]. In addition, the amount of TG present in hepatocytes represents a complex interaction among these 4 sources and fatty acid oxidation (FAO) and FA export within very low-density lipoprotein (VLDL)-TG [2].

1.1.1 Lipid absorption by the liver

The lipids taken up by liver may derive from the diet followed by the transport in the circulation, which requires specific transporters. Free fatty acids are very poorly soluble in the water and most of the fatty acids in plasma are transported loosely bound to the plasma protein albumin [3]. While dietary lipids in the form of triacylglycerol (TG) are transported by chylomicrons and very low density lipoprotein (VLDL) [4]. Chylomicrons are the largest and least dense lipoprotein particles found in plasma and contain the highest proportion (about 85%) of triacylglycerol. They are derived from dietary sources of triacylglycerol

only, are produced by intestinal epithelia, and are delivered to the circulation via the lymphatic system [5].

Circulating triacylglycerols (in the form of chylomicrons or VLDL) are not taken up directly into the cells by the liver or any other organ. The triacylglycerol molecules must first by hydrolyzed enzymatically outside the cell to fatty acids and glycerol; this is carried out by lipoprotein lipase, which is attached to the outer surface of e.g. hepatocyte [6]. In the liver, specific transporters (FAT and L-FABP) are involved in the uptake and intracellular traffic of these molecules [7]. The hepatocyte then converts TG to diacylglycerol (carried out by microsomal lipase) and then to fatty acids, which are then activated and combined with coenzyme A, which allows their transport into the reticulum luminal space by intraluminal carnitine acyltransferase, where they are again re-esterified by TG diacylglycerol acyltransferase 2, and become a part of nascent hepatic VLDL, or are stored within lipid droplets [6]. VLDLs are similar in composition to chylomicrons but considerably smaller in size. Although chylomicrons carry up to 80% of the TAG during the post-prandial period they are significantly outnumbered by the smaller VLDL particles at all times [8]. Although VLDL receptor mRNA is mainly expressed in the muscle, heart , adipose tissue [9], VLDL can still be taken up by the liver through VLDL receptor, forming a route of re-absorption of VLDL formed in the liver itself [10]. Non-esterified fatty acids (NEFA of FFA) enter cells via transporters (fatty acid transport protein (FATP) or fatty acid translocase (FAT), CD36) or diffusion [11]. Non-esterified fatty acids and fatty acyl-CoA are bound to FABP and acyl CoA binding protein which transport them to intracellular compartments (for export or oxidation) or the nucleus (to be used as signalling molecules for transcription

factors) [12]. Cells exposed to exogenous fatty acids rapidly assimilate the fatty acids into neutral and polar lipids, and part of the fatty acids pool is oxidized. The result of these metabolic pathways leads to low levels of intracellular NEFA and fatty acyl-CoA [13].

1.1.2 *De novo* synthesis of fatty acids.

The liver and the adipose are two major tissues that can produce fatty acids in the body. Fatty acids synthesized in the liver are destined for export through lipoprotein production, and thus provide an energy source and components needed for membrane structures building. In adipose tissue, *de novo* synthesis of fatty acids is aimed for fat deposition and long-term energy storage [14,15]. Synthesis of fatty acids occurs in the cytosol and it is an extension of an alkanoic chain, two carbons at a time, in which acetyl units (derived from either glucose or acetate) are added successively to acetyl-CoA. The rate-limiting step in this pathway is catalyzed by acetyl-CoA carboxylase A, which is considered to be the chain extender substrate (donor of acetyl units) in the elongation process. Formation of a new C–C bond by condensation of the acetyl and malonyl moieties is coupled with an energetically favourable decarboxylation, so that the carbon originating from CO_2 introduced in the reaction catalyzed by acetyl- CoA carboxylase is recycled [16].

Activity of fatty acid synthase (FAS) a key multifunctional enzyme that catalyzes the entire pathway of palmitate synthesis is closely associated with the rate of fatty acid synthesis and its expression can serve as a marker of *in situ* lipogenesis [17,18,19]. Fatty acid synthase is expressed in the two major sites of fatty acid production in the body, liver and adipose tissue, but the

relative contribution of these sites to de-novo lipogenesis is species dependent with liver in humans being the main organ for fatty acid synthesis while in rodent both liver and adipose tissue being equally important [13]. FAS is regulated positively by insulin and nutritional state (abundance of carbohydrates) [18], whereas glucagon and catecholamines inhibit its activity. Increased concentration of fatty acyl-CoA in the cytosol also inhibits the acetyl-CoA carboxylation. Regulation of FAS is also largely determined by intracellular fatty acid concentration (especially polyunsaturated fatty acids), an increase of which lowers FAS activity [20]. The regulation of lipogenic gene expression by insulin and fatty acids is mainly mediated by transcription factors, such as sterol regulatory element binding proteins SREBPs and in part by nuclear receptors such as liver X receptors (LXRs) [21].

1.1.3 Fatty acid oxidation in the liver

Fatty acid oxidation occurs in mitochondria, peroxisomes and microsomes. The β-oxidation occurs in the mitochondria and the peroxisomes, whereas ω-oxidation occurs in the microsomes [22]. Carnitine palmitoyltransferase-1 (CPT1), the rate limiting step of mitochondrial fatty acid β-oxidation in cells, is located in the outer mitochondrial membrane. CPT1 controls the transport of long-chain acyl-CoA into the mitochondria [23,24]. Mitochondrial oxidation may be either complete or incomplete. During incomplete oxidation ketone bodies are being formed [25]. Ketogenesis therefore allows the liver to metabolize about five times more fatty acids (for the same ATP yield), and conversion of fatty acids into water-soluble particles is a short-term way for redistribution of energy [10,26]. Ketogenesis is controlled

indirectly by CPT1 and directly by the activity of the mitochondrial key regulatory enzyme 3-hydroxy-3-methylglutaryl-CoA synthase (HMG-CoAs), which also plays important role in cholesterol synthesis [27]. There are differences between the peroxisomal and mitochondrial fatty acid oxidation. In peroxisomes, very long chain fatty acids are being oxidised, while mitochondrial β-oxidation is responsible for the oxidation of short, medium and long chain fatty acids [13].

1.1.4 Triacylglycerol export (VLDL turnover)

The triglycerides are bound to apolipoprotein B (Apo B) to form the mature lipoprotein particle to be secreted as VLDL. Apo B synthesis is stimulated by elevated FFA and TG levels, as well as by the microsomal transfer protein (MTP), whereas it is inhibited by insulin [28]. Elevated FFA afflux to the liver, and normal or decreased insulin concentrations stimulate secretion of mature VLDL-Apo B. The majority of triglyceride incorporated into VLDL originate from intracellular storage pools rather than from *de novo* synthesis [29]. For example, in obese mice, *de novo* lipogenesis in the liver does not stimulate VLDL output [30], whereas in rats, high carbohydrate diets enhance secretion of VLDL-triacylglycerols, but this increased hepatic output of triacylglycerols is accomplished by enhanced formation of VLDL triglyceride from exogenous NEFA rather than from fatty acids synthesis in the liver [28].

1.1.5 Alterations in hepatic lipid and glucose metabolism

In last few decades, obesity has risen to considerable proportions in Western societies. It is present in literally all age and socioeconomic groups. It is a complex condition produced and accompanied by many factors, which high

calorie diet and lack of exercise are most significant [31]. Obesity results as a direct imbalance between energy intake and energy expenditure. The excess of calories is principally stored in adipose tissue in form of triglyceride. However, obesity often leads to storage of the fat in tissues other then adipose tissue such as liver and skeletal muscle. Obesity and the metabolic syndrome promote alterations in hepatic lipid and glucose metabolism and are linked to formation and pathogenesis of fatty liver disease [32,33,34].

1.2 Features of non-alcoholic fatty liver disease.

1.2.1 Characterization of the fatty liver disease.

Liver abnormality known as non-alcoholic fatty liver disease (NAFLD) is characterized by an increase in intrahepatic triglyceride (IHTG) content (steatosis) with or without inflammation (steatohepatitis) or fibrotic components (cirrhosis). In the past, excess alcohol consumption was the primary reason for the majority of cases of NAFLD, but in recent years, non-alcoholic causes of NAFLD have attracted considerable attention [35,36,37]. Both alcoholic fatty liver disease (AFLD) and NAFLD generally begin as simple hepatic steatosis, and if the cause persists, this steatosis subsequently progresses to steatohepatitis, cirrhosis, and possibly to liver cancer [34]. Hepatic steatosis is defined as excess accumulation of fat (triglyceride) in hepatic parenchymal cells (hepatocytes) of the liver, and its aetiology is usually diverse [38,39,40]. Morphologically, hepatic steatosis appears as accumulation of large (macrovesicular) or small (microvesicular) cytosolic fat droplets in liver

parenchymal cells. A patient can be diagnosed with hepatic steatosis when lipid content in the liver exceeds 5–10% by weight [41,42]. In most cases hepatic steatosis is macrovesicular in type in the alcoholic, obese, and diabetic states. Macrovesicular steatosis, appears when hepatocytes contain a large, single vacuole of fat, which fills the cytosol and pushes the nucleus to the periphery (Fig. 1.1A). In microvesicular steatosis, hepatocytes are occupied by numerous small lipid droplets that do not displace the centrally located nucleus (Fig. 1.1B) [23].

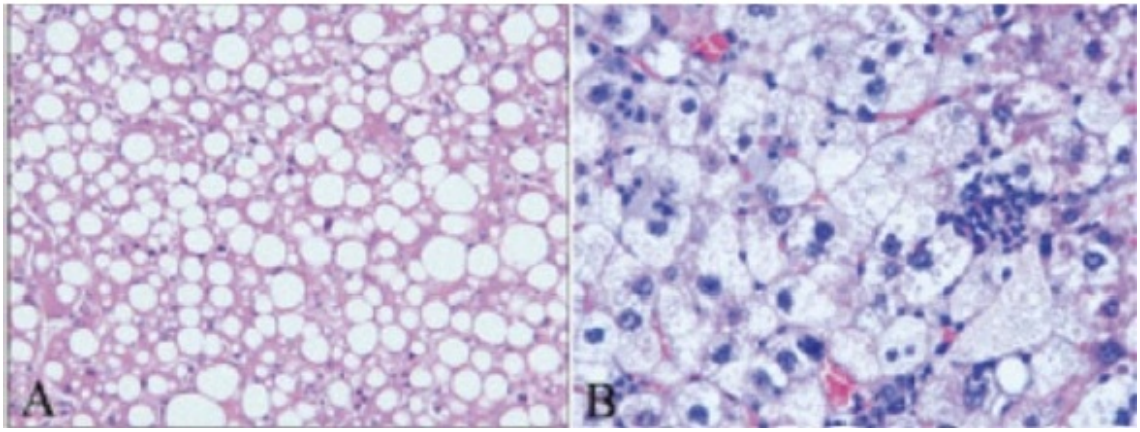

Figure 1.1 Histopathological features of non-alcoholic fatty liver disease. Human liver biopsies from: A: macrovesicular hepatic steatosis with a large lipid vacuole in the hepatocyte. B: microvesicular steatosis where small fat droplets fill the hepatocyte cytoplasm with a central or peripheral location of the nucleus (stained blue). Adapted from Reddy et al, 2006.

Generally genetic or toxin-related abnormalities in mitochondrial and peroxisomal β-oxidation of fatty acids might induce microvesicular hepatic steatosis, and this type tends to be rapidly progressive and more severe. Some hepatocytes with microvesicular steatosis may also reveal a macrovesicular fatty change, suggesting that the progression of disease can lead to fusion of

small lipid vacuoles to become large droplet [43]. In AFLD and longstanding NAFLD, hepatic steatosis is generally macrovesicular and, but in some cases, the phenotype of the droplets might be intermediate [34,44].

The steatotic liver is vulnerable to secondary insults, which lead to hepatocellular inflammation and fibrosis. However, the exact cause of progression to non-alcoholic steatohepatitis (NASH) is not entirely defined. Currently the leading theory is that an in initial insult leads to fatty deposition in hepatocytes, while a second mechanism triggers the inflammation that targets lipid-loaded hepatocytes (steatohepatitis). This is known as two hit hypothesis [45]. A variety of endogenous and exogenous factors have been implicated to produce a second "hit," including hormones derived from adipose tissue (like adipocytokines), oxidative stress or bacterial-borne endotoxin [45]. A small subset of patients with NAFLD (5-10%) develop NASH, which can potentially progress to more serious liver injury ending in liver cirrhosis. However, the occurence of NAFLD is nearly 50% in people with diabetes, 76% in those with obesity, and 100% morbidly obese with diabetes [41]. Moreover, older patients and those with hypertension and diagnosed obesity are at greater risk of developing NASH [32].

1.2.2 Non-alcoholic steatohepatitis.

NASH has been defined as part of the spectrum of fatty liver disease, but the characteristic distinguishing feature is necroinflamatory damage to the tissue. In the diagnosis of NASH, a daily alcohol intake exceeding 20 g/day must be excluded due to the known hepatotoxicity of such alcohol use [32]. Hepatic injury is characterized in NASH by hepatocyte necrosis and

inflammatory infiltrates. Unlike NAFLD, NASH is a progressive liver disease, and if it is not reversed might lead to cirrhosis, liver failure and need for liver transplantation [34]. Histologic features of NASH are virtually very close to those seen in alcoholic steatohepatitis (although some differences exist like sclerosing hyaline necrosis, the veno-occlusive lesion first described and alcoholic foamy degeneration) [34]. And lipid peroxidation and oxidative stress appears to be responsible from the production of reactive oxygen species and inflammation in NASH [23]. Histological markers used to distinguish NASH from fatty acids accumulations vary in the literature, but when diagnosing NASH based on histopathology, degree of steatosis, the presence of hepatocellular ballooning and the lobular and portal inflammation is usually assessed. Typical look of NASH diagnosed liver biopsy is shown on Fig. 1.2.

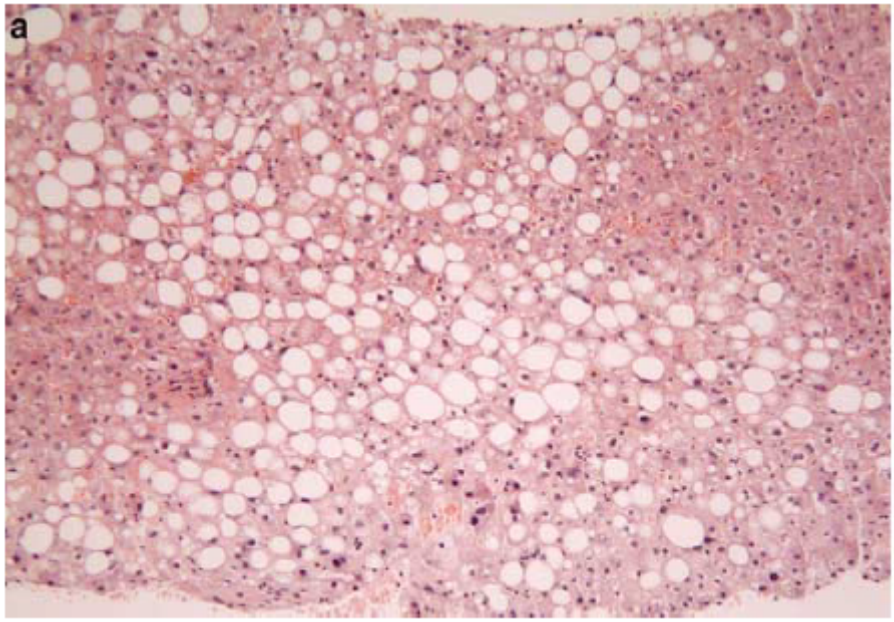

Fig 1.2 Characteristic features of non-alcoholic steatohepatitis. Zone 3 accentuation of the lesions of steatosis (large and small droplet type), ballooning and lobular inflammation. Adapted from Brunt E. 2007.

1.2.3 Liver cirrhosis

If the causes persist, NASH may progress to later stages of liver disease with fibrosis and cirrhosis. This medical condition is characterized by replacement of functional hepatocytes by fibrotic, scar tissue, which greatly weakens liver functionality. Although a some regression has been shown in animal models, cirrhosis is generally thought to be irreversible, and treatment usually focuses on stopping progression and disease-associated complications [46]. However, fibrosis progression in patients with NAFLD appears to be slow, sometimes it might take several decades for developing liver cirrhosis [42]. Currently, cirrhosis is being diagnosed primarily on histological evidence of late stage fibrosis showing the end stage of the wound healing process, but without adequately signifying the complexity of its pathogenesis [47]. Histopathology of the fibrotic changes in the liver is shown in Fig. 1.3.

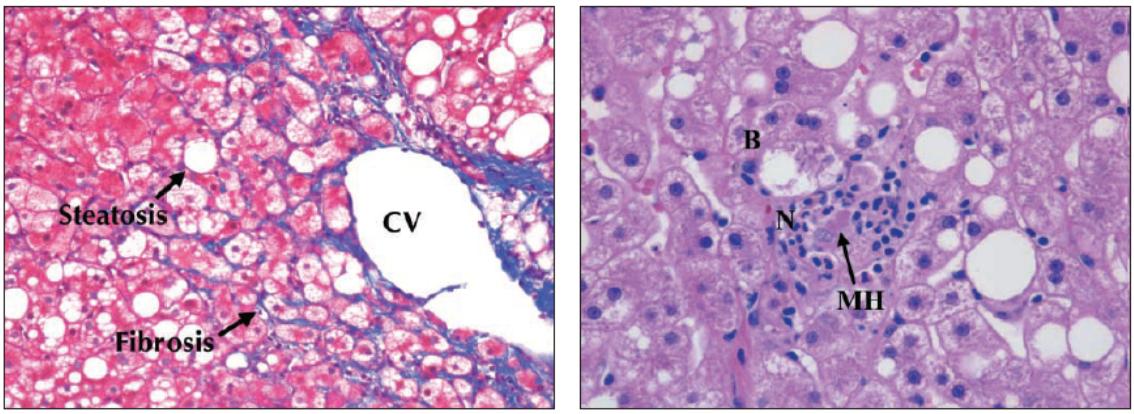

Figure 1.3 Fibrosis in non-alcoholic steatohepatitis (NASH). Left: trichrome stain of liver showing both macrovesicular lipid droplets and fibrosis, most prominent in zone 3 near the central vein (CV). Right: Necrosis and inflammation in NASH. Hematoxylin and eosin stain of liver with injured ballooned hepatocytes (B) and mild infiltration of neutrophiles (N). Adapted from Adams et al 2005.

1.2.4 Liver carcinoma

The last stage that NAFLD can turn into is development of hepatocellular carcinoma (HCC). Primary liver carcinoma including both HCC and intrahepatic cholangiocarcinoma often occur in patients with NASH, especially in those with advanced fibrosis and cirrhosis, and the occurrence of HCC is strong predictor of death in patients with old age and well developed liver fibrosis [48]. It is worth mentioning that obesity and NAFLD alone can induce proliferation and decrease the rate of apoptosis in hepatocytes in a mouse model, resulting in hepatic hyperplasia also in the absence of inflammation or cirrhosis [49].

1.2.5 Clinical characteristics of NAFLD

NAFLD and NASH is usually an asymptomatic condition [32]. First the exact prevalence of both NAFLD and NASH is unclear because confirmation of the diagnosis requires liver biopsy. For obvious ethical consideration, no large screening studies have used this invasive technique. However, liver biochemistry abnormalities are common, and usually drag attention where otherwise unexplained elevations in liver tests or abnormal hepatic imaging (e.g. incidental finding) are found [33]. Although, fatty change of the liver can be well characterized by ultrasonography [50], computed topographic scanning [33] or magnetic resonance imaging [35,51] it does not differentiate NAFLD from NASH, and therefore it cannot replace biopsy-based liver histology. Recently many studies were undertaken to discover and evaluate non-invasive markers for NAFLD [52]. Serum-derived gene expression and proteomic markers attract attention along with fibrosis and apoptosis biomarkers allowing distinguishing between stages of fatty liver disease. In order to assess the utility of a

combination of individual markers in diagnosing and staging various forms of NAFLD a range of useful algorithm tests has been also proposed [52].

1.3 Causes of non-alcoholic fatty liver disease

A reasonable correlation has been found between the risk for NAFLD or NASH and increasing body weight [53,54]. However, non-obese people have also a risk of developing non-alcoholic fatty liver disease. The risk factors for NAFLD among people who are not obese and in non-diabetics are: impaired fasting glycemia, hypertriglyceridemia, hyperuricemia, hypertension or levels of high-density lipoprotein cholesterol (HDL) [41] and to some extent genetics [55] and others causes [56]. In the next part of this introduction some of the most common causes of NAFLD will be shortly characterized.

1.3.1 Nutrition and *de novo* lipogenesis.

Intake of the dietary calories is closely linked with *de novo* fatty acids in the liver. After the meal, both ingested fat and *de novo* lipogenesis increase and can provide more than 50% of the fatty acids entering the liver [57]. Dietary glucose and fat are important regulators of lipogenesis via activation of SREBP-1c. Glucose also is an activator of carbohydrate response element-binding protein (ChREBP), which exerts, similarly to SREBP-1c, stimulatory effects on the expression of genes involved in lipogenesis and triglyceride synthesis [58]. Therefore during fasting lipogenesis accounts for less than 5% of hepatic fatty acids supply in healthy subjects, but lipogenesis may substantially increase in subjects with fatty liver creating positive feedback loop [18]. Expression of lipogenic gene SREBP-1c also depends on saturation of fatty acids. Saturated

and trans-unsaturated FFAs are known to increase and mono and polyunsaturated FFAs decrease its expression and activity [21,24]. This was confirmed in human studies, where individuals with fatty liver had higher intake of calories as well as saturated fat and cholesterol when compared with healthy controls. This was confirmed in human studies, where individuals with fatty liver had higher intake of calories as well as saturated fat and cholesterol when compared with healthy controls. Apart from that, they also have lower intake of polyunsaturated fatty acids, fibre, and antioxidant vitamins [59,60]. Some studies also suggest, that iron overload can play some role in pathogenesis of in NASH [32]. Additionally there are studies showing that low carbohydrate, ketogenic diet is correlated with a greater weight loss, a better lipid profile, and clearly improved steatosis than low fat diets [61]. Among the carbohydrates, fructose appears to have the strongest effects on lipogenesis [62]. Because use and consumption of high fructose corn syrup has massively increased in the last decades in the western world, its contribution to the increase of the occurrence of fatty liver disease might be significant [63].

1.3.2 Body fat composition and liver fatty acids influx.

Liver fat measured by proton magnetic resonance spectroscopy (^{1}H-MRS) is positively correlated with total adiposity expressed as body mass index (BMI) or percentage body fat. Furthermore, the association of liver fat with visceral adiposity, measured as waist circumference, is also positive [38]. However according to some studies visceral adipose index (VAI) cannot be adequate predictor of liver histology in patients with NAFLD [35]. A proposed

mechanism explaining the relationship of overall and visceral obesity with liver fat is as follows. When adipose tissue expands, macrophages infiltrate it and eventually adipose tissue is highly exposed to proinflammatory cytokines and probably, therefore, can become insulin resistant [64]. In normal healthy condition insulin mediates suppression of lipolysis [15]. When the tissue is non-responsive to insulin, it increases release of free fatty acids (FFAs) from adipocytes. In this case visceral adipose tissue (VAT) is more important than subcutaneous adipose tissue because it is metabolically more active [65]. Additionally, increased lipolysis in VAT is thought to result in an elevated flux of FFAs directly into the portal vein and therefore to the liver, a physiological phenomenon that is commonly defined as the “portal hypothesis” [66]. FFAs are then taken up by the hepatocytes. However, during fasting the main origin of the FFAs in the systemic circulation is considered to be subcutaneous fat [33]. Although in the fasting state liver fatty acids are taken up predominantly from the systemic plasma FFA pool, the portal FFA route of supply to the liver may be more relevant postprandially as in obese individuals state of fasting is of course not the dominant one. Another factor associated with overgrown adipose tissue, which contributes to NAFLD is inflamed adipose tissue in obesity. High levels of proinflammatory cytokines as TNF-α and interleukins, particularly IL-6, suppresses the production of the insulin-sensitizing adiponectin [67]. Therefore, the imbalance in the secretion pattern of these adipocytokines is considered to represent another link between obesity and fatty liver. Circulating adiponectin closely and reversely correlates with liver fat content and hepatic insulin resistance [68]. Apart from hypothalamic effects that leptin has in the regulation of food intake, this adipokine is considered another important regulator of liver

fat. Although the mechanisms of the protective effect are not fully understood, the most probable explanation of leptin anti-steatotic effects lies in enhancing lipid oxidation and inhibiting lipogenesis in liver tissue [69]. In the animal studies where recombinant adenovirus-receptor constructs containing the normal leptin receptor were injected into obese Zucker diabetic rats, the marked hepatic triglyceride reduction was observed. It is known that most of the adenovirus-receptor constructs is taken up by the liver [69].

Surprisingly the reverse situation, where the total body adiposity is very low, can also result in ectopic lipid accumulation including liver steatosis. The lipoatrophy is a group of syndromes with various aetiology, characterised by paucity of adipose tissue. In lipoatrophy, adipocytes can by non-functional or normally functioning but lacking lipids to be stored. The latter situation reflects conditions such as extreme exercise, starvation, or treatment with pharmacological doses of leptin [55]. However, when adipocytes are present but non-functional, e.g. caused by autoimmune mechanisms or defects in adipogenesis, it results in ectopic fat accumulation as a way the body's attempt to compensate for the overall lack of storage sites for lipids. Therefore, hepatic steatosis is uniformly present in severely affected patients, some of whom develop steatohepatitis and cirrhosis [55,70].

1.3.3 Decreased hepatic lipid export

Microsomal triglyceride transfer protein (MTP) mediates TAG association with apolipoprotein B (ApoB). Inhibition of MTP in mice results in inefficient lipid attachment to ApoB, which leads to impaired TAG transfer out of the liver [71]. Additionally, hyperinsulinaemia can alter the synthesis of ApoB, leading to

decreased VLDL production. This finding was also confirmed in some of the NASH patients who were reported to have tempered synthesis of ApoB, which almost certainly contributes to hepatic fat accumulation [72]. However, in the subjects with high intrahepatic TG content, apart from having impaired insulin action in liver, they also have elevated hepatic VLDL-TG secretion rate. Moreover, intrahepatic TG content is the best independent predictor of increased VLDL-TG secretion rate [73].

1.3.4 Defects in the liver fat oxidation

Some reports indicate that physical activity can be negatively associated with liver fat, and this effect is independent of BMI [53]. Mechanisms linking fitness and liver fat most likely include factors regulating hepatic lipid oxidation. Fitness is known to cause enlargement of and increase in mitochondria in skeletal muscle and the generation of type I fibers [74]. Mitochondria play an essential role in hepatocyte metabolism, representing the primary site for the oxidation of fatty acids. Hepatocytes are particularly rich in mitochondria, occupying approximately 18% of the liver cell volume [75]. Disruption of the straight-chain acyl-CoA oxidase gene in the mouse leads to the development of severe hepatic steatosis. These animals accumulate high levels of very long-chain fatty acids (>C22) in plasma and hepatomegaly with accompanied by steatohepatitis. In later stages, hepatocyte death and hepatocellular carcinomas develop in these mice [76]. Very long-chain fatty acids (>C20) are not processed by the mitochondrial β-oxidation system, and they require peroxisomal β-oxidation to shorten the chain length for further completion of oxidation in mitochondria [23]. The well known master of genes involved in liver

lipid oxidation is peroxisome proliferator activated receptor α (PPARα). Genetic ablation of the PPARα causes massive fatty acid accumulation in response to fasting in these animals [77]. PPARα-KO mice fail to up-regulate genes involved in fatty acid oxidation systems in the liver and are unable oxidize the influxed fatty acids and thus develop severe hepatic steatosis. Furthermore, the administration of PPARα agonists to rats with induced fatty liver by methionine and choline-deficient diet prevents development of steatohepatitis [78].

Summary of potential causes for NAFLD is shown on Fig 1.3

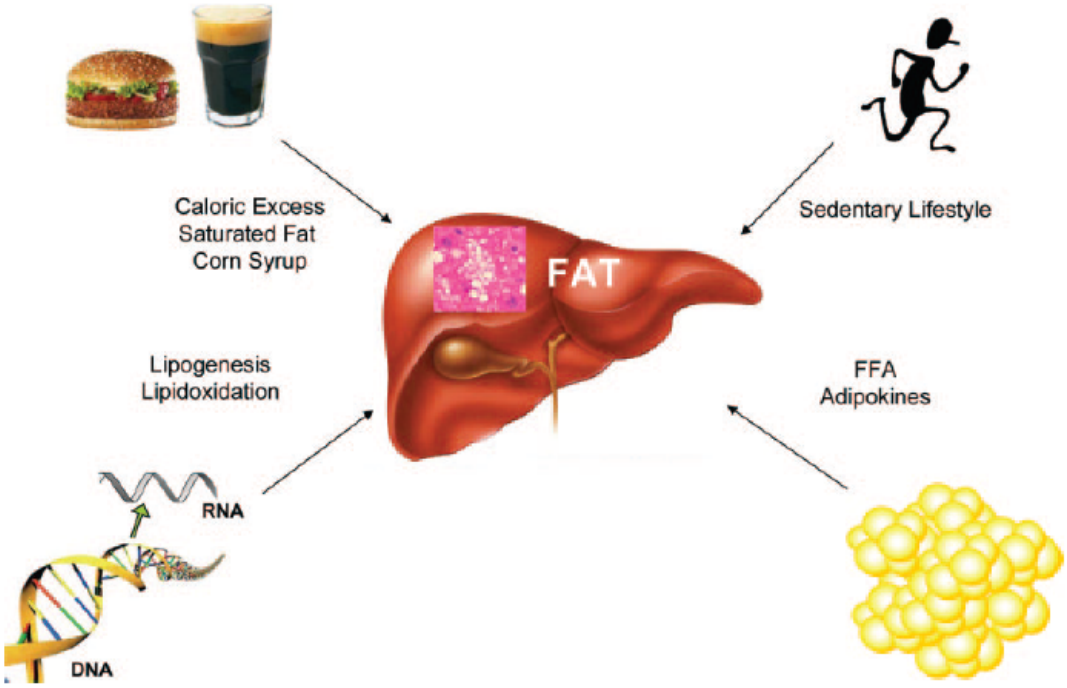

Figure 1.4 Major determinants of fatty liver. Adapted from Stefan et al, 2008

1.3.5 Treatment strategies for NAFLD

The aim of treatment for NAFLD is to slow the progression of the disease and to prevent liver-related illness and death. There are currently 3 types of approach to treat NAFLD: life style modification, pharmacology and liver transplantation. Liver transplantation may be necessary when the disease has progressed to cirrhosis and is complicated by liver failure or hepatocellular carcinoma. However, recurrence of steatosis after transplantation happens in 60%–100% of transplant recipients and progression to steatohepatitis is reported in one-third of cases [41]. Both weight loss and exercise are highly recommended for ameliorating some aspects of metabolic syndrome therefore these life style modifications are also useful for NAFLD treatment. Diets aim at reducing total daily energy intake, based on either a low processed carbohydrate, or low-fat diet, aimed at achieving about a 10% weight reduction. They should improve both metabolic and histopathological abnormalities in a diverse group of NAFLD patients. However, great care needs to be taken some studies suggested that too rapid a weight loss may worsen the histopathology of patients with NAFLD. This happens likely due to massive lipolysis of WAT and infflux of free fatty acids to the liver, where, in such a situation the liver's oxidative capabilities are exceeded [10,53]. Moderate exercise is also effective way of improvement in the metabolic profiles of patients with NAFLD [79].

Pharmacologic therapy of NAFLD is evolving. To relieve oxidative stress in NASH, powerful scavengers of free radicals were tried [32]. α-Tocopherol or vitamin E have an excellent safety profile and their use helped to lower the levels of aspartate transaminase (AST), alanine transaminase (ALT). Betaines are trimethyl amino acids derived either from choline or from the diet. Betaines are essential to form phosphatidyl choline (PC), a component of VLDL, which is

the key molecule to export lipids from liver. Administration of betaine improves the export of lipids from hepatocytes [80].

Given that insulin resistance is central to the pathogenesis of NAFLD, insulin sensitizing agents find their application for pharmacotherapy for NAFLD. Metformin an inhibitor of gluconeogenesis has shown some results in human trials where it helped to improve ALT parameters but not histology. And another antidiabetic agents statins, inhibitors of cholesterol synthesis may reduce hepatic fat content in patients with hyperlipidemia and NASH [79].

Other groups of drugs which have been successfully applied in treatment of metabolic syndrome are peroxisome proliferator activated receptors (PPAR) ligands. PPARα was partly mentioned in the context of its abilities to control fatty oxidation in the liver, and agonists for another PPAR member, peroxisome proliferator activated receptor gamma (PPARγ), like pioglitazone or rosiglitazone also have some success in ameliorating some parameters of NAFLD. Another member of PPAR family PPARδ, is one of the promising drug target known for its anti-obesity actions [81] and therefore it is thought that modulation of its activity with small molecule would have great potential for treating NAFLD.

1.4 PPARS as drug targets for NAFLD

1.4.1 PPARs as nuclear receptors

Superfamily of nuclear receptors includes endocrine receptors that mediate the actions of steroid hormones, thyroid hormones, and the fat-soluble vitamins A and D, but also a number of so called orphan nuclear receptors [82]. The name indicates that their activators, target genes, and physiological functions were initially unknown. With the progress of research, some of orphan receptors become "adopted" orphan receptors - with partially characterized ligands or target genes [83]. PPARs are examples of adopted orphan receptors. The structural organization of PPARs is similar to other nuclear receptors and consists of six regions. These proteins are build from an NH_2-terminal region that holds a ligand-independent transcriptional activation function (AF-1); core DNA-binding domain, containing two highly conserved zinc finger motifs that target the receptor to specific DNA sequences known as putative response element (PPRE) with the sequence AGGTCA N AGGTCA (direct repeat with a single nucleotide spacer (DR-1)) [84,85]. In the middle there is a hinge region that confers the protein flexibility needed for simultaneous receptor dimerization and DNA binding. And in the COOH-terminal region, ligand-binding domain (which also serves as dimerization interface) and a ligand-dependent activation function (AF-2) are localized. Typical structure of PPAR receptor is shown on Fig 1.4. X-ray crystal structures of the ligand binding domains exposed that PPARs contain a relatively large to other nuclear receptors hydrophobic-binding pocket. This large structural cavity is most likely the reflection of variety of putative ligands that can bind and activate PPARs [86,87].

Figure 1.5 Structure of PPAR receptor.

1.4.2 Characterization of PPARs

There are three closely related members of the family: PPARα (NR1C1), PPARβ/δ (NR1C2) and PPARγ (NR1C3). There are two forms of PPARγ, (γ1 and γ2, with differing amino termini) [88]. Messenger RNA of these three isoforms RNA's are transcribed from different genes: PPARα gene is located on chromosome 22 in the general region22q12-q13.1, the PPARγ gene is mapped on chromosome 3 at position 3p25, and the PPARδ is found on chromosome 6, at position 6p211.1-p21.2 [85]. Studies with fluorescently labelled PPARα and PPARγ fusion proteins show that PPARs are primarily found in the nucleus, independent of the presence of ligands [89]. PPARα is well expressed in metabolically active tissues such as liver, brown adipose tissue, muscle, and heart. PPARδ is ubiquitously expressed with high levels especially in skin, brain muscle and macrophages. PPARγ is most highly expressed in adipose tissue, yet reasonable levels of PPARγ mRNA can also be found in other organs including, colon, and especially lung [89].

Prior to DNA binding, PPARs form heterodimers with retinoid X receptors (RXR). The heterodimerization between PPARs and RXR has been experimentally demonstrated as ligand-independent. In living cells, PPARs can

efficiently heterodimerize with RXR in both absence and presence of ligand [88]. In the absence of the ligand, PPAR-RXR heterodimers are actively repressed by recruitment of co-repressor complex which possesses histone deacetylation activity (HDAC) and chromatin modifying factors, which results in silencing of the target genes [31]. Upon ligand binding, nuclear receptors undergo a conformational change that co-ordinately dissociates co-repressors and facilitates recruitment of co-activators proteins to enable transcriptional activation [88]. It is known that, the RXR ligand, 9-cis retinoic acid, enhances PPAR action. Retinoids may therefore modulate the action of peroxisome proliferators and also PPARs may interfere with retinoid action [90]. The peroxisome proliferator-activated receptors are activated by polyunsaturated fatty acids, eicosanoids, and various synthetic ligands [91]. However, their distinct expression patterns and gene knockout experiments have unravelled that each PPAR subtype performs a specific function in fatty acid homeostasis.

1.4.3 PPARα function.

The transcription factor PPARα modulates metabolism through activation of a set of target genes in a variety of metabolically active tissues, particularly under fasting conditions. Cross-species prognostics are not always possible due to differences in metabolism or expression levels. Generally, the effect of activation by the PPARα synthetic agonist WY14643 is more pronounced in mice than in humans [92,93,94]. Long-term treatment of rodent with peroxisome proliferators like fibrates or WY14643, results in hepatomegaly and in increased incidence of hepatocellular carcinomas, which is not found in humans. Ablation

of PPARα immunises mouse to fibrates-related effects in the liver, but in the same time makes them prone to fasting-induced hepatic triglyceride accumulation [95]. However, in both species PPARα regulates many identical gene ontology classes, including lipid metabolism. Genes encoding for mitochondrial proteins of the β-oxidation pathway are induced by PPARα activation, such as acyl-CoA synthetase (Acs) coding for an enzyme which activates FA to their fatty acyl-CoA derivatives. Also genes of the short, medium, long and very long-chain acyl-CoA dehydrogenases coding for proteins that catalyze the first step in FA oxidation in a chain length-specific manner, are under the control of PPARα. PPARα also has important role in governing fatty acid oxidation in perixosomes. The first characterized PPARα target gene, acyl-CoA oxidase 1 (Acox1) encodes the rate-limiting enzyme of this process in perixosomes [96]. Peroxisomal β-oxidation becomes increasingly important during periods of increased delivery of fatty acids into the liver, which is typical in NAFLD. In addition to mitochondrial and peroxisomal β- oxidation, ω-hydroxylation occurs in smooth endoplasmic reticulum. In both mice and humans activation of PPARα modulates the expression of cytochrome P450 4A genes. CYP4A enzymes, such as the human enzyme CYP4A11, catalyse the ω-hydroxylation of medium and long-chain fatty acids [97]. Other crucial processes regulated by PPARα activation are lipoprotein synthesis and assembly. The VLDL abundance in plasma is influenced by lipoprotein lipase (LPL) activity in peripheral cells. The hepatic expression of this hydrolase, which is required for VLDL triglyceride particles lipolysis, is up-regulated by PPARα [98]. Additionally, activity of LPL is stimulated by Apolipoprotein A5 (ApoA5) and

inhibited by ApoC3. Activation of PPARα increases ApoA5 and decreases ApoC3 mRNA, which results in a plasma TAG lowering effect [98].

1.4.4 PPARα in treatment therapy for NAFLD

Pharmacological management of NAFLD by PPARα action benefits mainly from its ability of promoting fat burning in the liver and ameliorating general aspects of type 2 diabetes and obesity-related problems. Although natural PPARα ligand was recently identified [17], clinically utilized PPARα agonists belong to fibrates class such as gemfibrozil, clofibrate, fenofibrate and ciprofibrate, which have been used for decades in the treatment of dyslipidemia. This abnormality is characterized by low HDL and high TG levels. The ability of PPARα to increase HDL levels comes as a result of the stimulation of expression of ApoAI and ApoA2 expression, the major apolipoproteins of HDL [99]. However, fibrates are weak agonists of PPARα (EC_{50} values 20-500μM), and high doses are required for effective treatment [100]. On the other hand, fibrates are considered as safe drugs, with few side effects, such as an increase in myopathy, and possible lithogenicity rarely reported in humans.

Animal studies show that PPARα-KO mice fed the methionine and choline-deficient diet (MCD) display greater levels of steatohepatitis compared to wild-type mice on the same diet. And delivery of the PPARα agonist WY14643 reduces or even reverses MCD-induced steatohepatitis and fibrosis in wild-type mice [101]. Apart from promoting lipid oxidation, PPARα activation also exhibits anti-inflammatory effects via inhibition of IL-6 and C-reactive protein. These effects are at least in part mediated by reduced expression of

NF-κB as well as an increase in IκB-R leading to prevention of NF-κB nuclear translocation [102]. This property of PPARα signalling might be of particular interests when liver steatosis progresses to NASH stage. In addition to regulating fat oxidation and lipoproteins turnover, PPARα influences glucose homeostasis. Under fasting conditions, PPARα-KO mice apart from hepatic lipid accumulation, suffer from hypoglycaemia and hyperinsulinaemia. In these animals glucose synthesis from lactate and lactate production, are strongly reduced, whereas the hyperinsulinemia found in fasted PPARα-KO animals results from impaired repression of insulin secretion in the pancreas [103].

1.4.5 PPARγ as a drug target for NAFLD

PPARγ can be activated by various fatty acids, prostaglandins, arachidonic acid metabolites or components of oxidized LDLs. However, the affinity of the receptor for many of these ligands is low [104].

Target genes of PPAR-γ include the adipocyte fatty acid binding protein, lipoprotein lipase, the uncoupling protein UCP1, the scavenger receptor CD36 [104]. In contrast to PPARα, PPARγ activation promotes fat storage by increasing adipocyte differentiation and induction of a number of important proteins involved in lipogenesis [105]. This subsequently leads to increased fatty acid uptake by adipose tissue cells. PPARγ is the important molecular target for drugs of the thiazolidinedione (TZDs) or glitazones class [105]. These drugs sensitize cells to insulin and therefore are in use for their anti-diabetic effects in the liver, adipose tissue and skeletal muscle [99]. On the other hand,

mice with PPARγ adipose-specific knockout are insulin-resistant and unresponsive to TZDs [106].

TZD treatment leads to adipose tissue remodelling. The mechanism consists of two physiological events. First is selective pre-adipocyte differentiation in subcutaneous depots and second is promotion of apoptosis of older and larger insulin-resistant visceral adipocytes. The new adipocytes are smaller in size, they are more sensitive to insulin and therefore can take up more fats [107]. Activation of PPARγ by TZDs enhances lipolysis of circulating TGs by lipoprotein lipase and their storage in adipose tissue. Furthermore, TZDs stimulate the use of glycerol for TG production, thereby reducing FFA release from adipocytes. This reduction in FFAs removes the burden of fatty acids in skeletal muscle, liver and pancreas, leading to a reduction in hepatic glucose production. This hypoglycemic effects of TZDs improves glucose utilization in skeletal muscle [108].

The important overall therapeutic effect of PPARγ activation in NAFLD is thus promotion of "new" storage space in adipocytes. It helps to relieve other organs from excessive fatty acids load. An increase in insulin sensitivity and glycemic control, coupled with a reduction in circulating free fatty acids helps to ameliorate type 2 diabetes-associated abnormalities.

Additional function of PPARγ in the aspects of treatment of NAFLD emerges from macrophage biology. The physiological consequences of macrophage-specific deletion of PPARγ in BALB/c mice are increased insulin resistance in skeletal muscle and liver, and exacerbation of diet-induced obesity. In the absence of functional PPARγ, macrophages cannot appropriately suppress inflammatory cytokine production [109]. This is particularly important

taking under consideration that increased systemic levels of pro-inflammatory cytokines can speed up progression from simple steatosis to NASH. Other studies also confirm that administration of PPARγ ligands can ameliorate inflammatory responses in multiple organs [104].

Hepatic stellate cells play role in NASH progression to cirrhosis by promoting hepatic fibrogenesis after transdifferentiation [110]. PPARγ activation was also found to preserve hepatic stellate cells in dormancy and thus prevent or even reverse hepatic fibrosis. The possible mechanism for this phenomenon might be the fact that the quiescent stellate cell has cytoplasm rich in lipids and thus shares some phenotypic features of adipocytes [111].

Despite being extensively used in type 2 diabetes patients, TZDs are not free of adverse effects such as pulmonary oedema and weight gain (2–3 kg per 1% glycosylated haemoglobin that is lowered), anaemia, congestive cardiac failure, or increased risk of myocardial infarction or bladder cancer [106]. Bearing in mind the dominant role of PPARγ in adipocyte formation, it is not surprising that its agonists promote weight gain. Therefore treatment with PPARγ ligands cannot be considered as a preferred long-term treatment in NAFLD. The best pharmacological agent in NAFLD management would promote fatty acids burning and utilization and would exhibit action in multiple organs. The last member of PPAR family, PPARδ is not yet targeted by any drugs on the market, however the recent extensive research indicates great potential of PPARδ selective agonists in treatment of fatty liver disease.

1.4.6 Functions of PPARδ receptor

PPARδ has a relatively large (1300 $Å^3$) in comparison to other nuclear receptors ligand binding pocket [112]. It is believed that this large pocket can bind easily various sizes of fatty acids and others amphipathic acids through hydrophobic interactions and hydrogen bond. Natural or synthetic eicosanoids (twenty-carbon essential fatty acids) like prostaglandin A1 or carbaprostacylin can also serve as PPARδ activators [113]. Although the exact natural ligands of PPARδ are so far unknown, the discovery of many synthetic activators (like GW501516), which serve as a potent full agonists, has allowed investigations on the functions of this receptor to progress [87].

Generally, it is thought that PPARδ is involved in lipid metabolism [74,114]. In animal models, most of the PPARδ-/- embryos die at an early stage due to placental and myelination defects and small numbers of survivors exhibits a reduction in fat mass/adiposity [85]. However, no change in fat mass is seen in adipose tissue specific knockouts of PPARδ [115]. On the other hand, in one study, a construct involving a viral fusion protein (VP16-PPARδ) was made and it turned out to be a potent ligand-independent transactivator of PPARδ target genes. When constitutively expressed in adipose tissue in murine model, it resulted in 40% decrease in the reproductive white fat depot [81]. Adipose tissue under expression of the transgene shows morphological changes: adipocytes are smaller and heterogeneous in size indicating that the reduction in total mass may results in fall of triglyceride accumulation. Therefore, it suggests that activation of PPARδ in adipose tissue promotes fatty acids combustion [81,116]. This indicates that the physiological actions of this

receptor may be complex [22]. PPARδ was also shown that it plays an important role in epidermal maturation and skin wound healing and could even serve as drug target for psoriasis [117,118,119].

It is known that exercise mediates up-regulation of PPARδ and creates a requirement for external or serum derived source of triacylglycerol as an energy substrate. It suggests induction of modification of lipoproteins by PPARδ activity [120]. There is a tendency for HDL cholesterol to drop in lack of physical exercise and vice versa: increase with the exercise. The administration of synthetic PPARδ ligands can mimics the exercise rise in the HDL levels while lowering triglyceride levels in animal models [81]. Muscle is a major place of metabolism of glucose and fatty acid oxidation. It is also an important regulator of cholesterol homeostasis and HDL levels. Muscle is also a major site of expression for PPARδ [22,121]. This nuclear receptor is predominantly found in oxidative rather than glycolytic myofibres. Some of the studies have demonstrated that constitutive activation of PPARδ can increase the number of oxidative myofibres and slow atherosclerotic lesion progression in obese dyslipidaemic rhesus monkeys [122]. Tanaka et al showed that activation of PPARδ in skeletal muscle by the selective, synthetic ligand (GW501516) increases the expression of the molecules involved in fatty acid oxidation, energy expenditure, related to fatty acid oxidation, mitochondrial respiration, oxidative metabolism and slow-twitch contractile apparatus as well as in adaptive thermogenesis [123]. The GW501516 treatment therefore improves fatty acid β-oxidation in the skeletal muscle, protects against diet-induced obesity, and improves glucose tolerance and insulin sensitivity [123,124].

PPARδ is also expressed in macrophages. However, the number of studies involving PPARδ and its role in inflammation is limited [125]. So far it has been shown that treatment with different PPARδ drugs promotes lipid accumulation in macrophages exposed to oxidized LDL or THP-1 cells exposed to serum [126]. This suggests promotion of atherosclerosis. On the other hand, in a study which low density lipoprotein receptor (LDLR)-/- mice were fed hypercholesterolemic diet with addition of PPARδ ligand has shown decrease in inflammatory gene expression when compared to controls. The main suppressed genes were: VCAM-1, MCP-1, IFN-γ, which are correlated with the development and progression of the arthrosclerosis [125]. Also study where treatment of THP-1 human monocytes with the highly-specific agonist (GW501616) showed both an increased level of expression of ABCA1, an important reverse cholesterol transporter, and stimulated apoAI specific cholesterol efflux [127].

1.4.7 PPARδ in hepatic lipid metabolism

Although in the liver PPARδ is not the most highly expressed member of PPAR family (eg PPARα protein is much more abundant than PPARδ in rodent liver), evidence is growing that it still play an important role in liver lipid metabolism.

In a study where db/db mice (model for diabetes with mutated leptin receptor) were treated GW501516 – high affinity PPARδ ligand, authors found that while GW501516 treatment significantly increased β-oxidation rate in muscle, 20% increase in liver triglyceride content was observed (cholesterol

and glycogen levels wwere not affected) [124]. It has been suggested that PPARδ regulates metabolic homeostasis through production of additional fat in the liver from the glucose to counterbalance fat burning in muscle. This work indicates that stimulation with PPARδ agonists gives control of the substrate utilization through regulation of diverse transcriptional programs in different tissues. Such metabolic shift resulting in reduction of hepatic glucose output improves insulin sensitivity [124]. Triglyceride increase following GW501516 stimulation may raise concerns if the long term drug treatment could be counter-productive and facilitate disease progression to NASH. However, there were no signs of fatty liver up to 6 months treatment in C57BL/6 mice [124]. Another work showing association of PPARδ activity with hepatic lipid deposition, utilized adenovirus-mediated liver restricted expression of PPARδ. The author found that although these mice exhibits reduced fasting glucose levels on chow or on high fat fed diet, this effect is accompanied by hepatic glycogen and lipid accumulation. However, despite observed elevated triglyceride levels, livers of these mice showed less damage and a reduction in JNK stress signalling [128].

On the other hand, in the study where mice fed a MCD diet (model of NASH) were given GW501516, treatment prevented the livers weights to be increased in comparison to MCD control mice [129]. It also decreased the numbers of lipid droplets when compared to MCD untreated animals and increased the levels of hepatic mRNAs associated with β-oxidation (acyl CoA oxidase, carnitine palmitoyltransferase-1, liver fatty acid binding protein and reduced the levels of a number of mRNAs associated with inflammatory response. In summary, in this study stimulation of PPARδ improved steatohepatitis [129].

Similarly, in a study done by Qin et al, it was shown that GW0742 and GW501516 treatment (both PPARδ agonists) of db/db mice markedly reduced the hepatocyte intracellular lipid accumulation. The proposed mechanism is as following: treatment induced the insulin-induced gene-1 (Insig-1), which is an endoplasmic reticulum (ER) protein retarding SREBP-1 activation at both the RNA and the protein levels. Inhibition of the processing the SREBP-1 into a mature form prevented the expression of lipogenic genes such as FAS. Results revealed direct binding of the PPARδ upstream of the transcription initiation site of Insig-1. It suggests that Insig-1 is a direct PPARδ target gene in hepatocytes [130]. Another study supporting a protective role for PPARδ showed that when Otsuka Long Evans Tokushima Fatty (OLETF) rats (rat model of type 2 diabetes) were treated with other PPARδ agonists GW0742 from 26 to 36 weeks a marked improvement in fatty infiltration of the liver occured. Authors also observed that GW0742 had inhibitory effects on palmitic acid-induced triglyceride accumulation and inflammatory markers in HepG2 and Raw264.7 cells [131].

Since there is contradicting evidence that PPARδ can either stimulate accumulation or promote disposal of fatty acids in mouse liver, a comprehensive understanding of its biological role in hepatic lipid metabolism is of vital importance.

1.5 Aims and objectives

PPARα and PPARγ have been proven as effective modulators of lipid metabolism and their ligands are used in clinical management of metabolic syndrome. Additionally, evidence implicating PPARδ as a key regulator of lipid homeostasis and glucose disposal is also growing. Although there are currently no available drugs targeting PPARδ, the compounds that selectively target PPARδ are being proposed to be used in obese, diabetic humans. However, the role of PPARδ signalling in liver lipid homeostasis is so far controversial with evidence both supporting and against the benefit of PPARδ agonism in treatment of the NAFLD. Therefore, we sought to test the hypothesis that PPARδ agonism may carry a risk of promoting hepatic fatty accumulation similar to Non-Alcoholic Fatty Liver Disease (NAFLD). The aim of this thesis was to characterise the hepatic lipid and transcriptional response using different strains of transgenic or knockout animal models and non-transgenic mice to selective PPARδ agonist and different diets. This project will therefore provide a solid understanding of the role of PPARδ agonists in hepatic and whole body lipid homeostasis, which can then be evaluated for potential risk of fatty liver toxicity in type 2 diabetic patients who may be treated with such agents.

Chapter 2 Materials and methods

2.1 Reagents used

Agarose gel running buffer (TAE) (50X)

242 g Tris base (2-amino-2-hydroxymethyl-propane-1,3-diol) (= 2 mole) was added to 500 ml of water and then 57.1 ml glacial acetic acid (= 100% acetic acid) (57.19 ml = 1 mole) was added followed up by addition of 100 ml 0.5 M Na_2 EDTA (pH 8.0) Buffer was made up to 1L with water

To prepare 0.5 M Na_2 EDTA (pH 8.0) 186.1 g of disodium ethylenediaminetetraacetate x $2H_2O$ was added to 800 ml of H2O. Mixture was stirred vigorously. Solution was then adjusted to pH 8.0 with NaOH (ca. 20 g of NaOH (pellets)). Hint: The disodium salt of EDTA does not go into solution until the pH of the solution is adjusted to ca. 8.0 by the addition of NaOH.

Add 20 ml of stock was mixed with 980 ml of water to get 1X solution.

Histology

Tissue fixing buffers (cryosectioning)

1% Paraformaldehyde/PBS/2mM $MgCl_2$:

2g Paraformaldehyde (PFA)

0.0812g $MgCl_2$

200 ml PBS

The mixture was to be warmed above 55°C (but temperature cannot exceed 60°C) to dissolve PFA.

Dehydration solution (30% sucrose):

0.0812g $MgCl_2$

60g sucrose

200 ml PBS

Glycerine Jelly Mounting Medium

Gelatin (Kitchen grade)	10 g
Distilled Water	60 ml
Glycerol	70 ml
Phenol	0.25 g

Gelatine was dissolved in the distilled water using sufficient heat to melt the gelatine, glycerol and phenol was then added. Mixture was mixed well and transferred to a small capped bottle and refrigerated.

1% Dextrin solution:

1g of dextrin was mixed in 100ml of sterile water (sterile glassware) gently heating the mixture (dextrin will not entirely go into the solution). Mixture was not autoclaved and not filtered. Solution was stored in 4°C.

DNA extraction buffers

Lysis buffer:

15ml NaCl salt solution (5M): 75mM

50ml EDTA (0.5M) ph 8.0: 25mM

100ml 10% (w/v) SDS: 1% (w/v)

The buffer was made up to 1L with 835 ml dH_2O

TE buffer

50 ml TrisCL (0.1M) ph 8.0: 10mM

1ml EDTA (0.5M) pH 8.0: 25mM

The buffer was made up to 500ml with 449 ml dH_2O

Lipid buffer

Lipid buffer used for dilution of liver lipid extracts was composed as follows:

Isopropanol : TritonX1000 : tert-butanol (2:1:1)

PPARδ agonist

PPARδ ligand:2-[2-methyl-4-([4-methyl-2-[4-(trifluoromethyl)phenyl)-1,3-thiazol-5-yl]methylsulfanyl]phenoxy]acetic acid (GW501516), was synthesised by AF ChemPharm Ltd, Sheffield, UK.

2.2 Animals

2.2.1 Mice used in experimental procedures

Non-transgenic C57BL/6 female mice were obtained from Harlan Laboratories (Harlan, UK). PPARα knockout mice (B6.129S4-*Ppara*^{tm1Gonz}/J) were purchased from Jackson laboratory (Bar Harbor, Maine, USA). PPARδ null animals were obtained from University of Lausanne, Switzerland, through the courtesy of Walter Wahli.

Human PPARδ transgenic mice were generated by cloning full-length human PPARδ downstream of the rat *CYP1A1* promoter. The PPARδ coding

sequence was amplified using primers PRMG15 (5´-CTA GTC TAG A**AT G**GA GCA GCC ACA GGA GGA AGC -3´) and PRMG3 (5´-CTA GTC TAG ATT AGT ACA TGT CCT TGT AGA TC TCC TG-3´), respectively (ATG start codon in bold). PCR products were cleaved with XbaI and cloned in plasmid pUHD10-3 (M. Gossen, unpublished, Genbank accession number U89931) creating pMGD7 (PPARδ). The integrity of the inserts was confirmed by sequencing and cleaved out using BamHI and ligated into plasmid pAHIR1-b-gal [132] cleaved with BglII, resulting in the plasmid pMGD72 (PPARδ). Proper insert orientation was confirmed by restriction endonuclease analysis and sequencing. Transgenic mice were generated by microinjection of the expression unit (NotI fragment) of the plasmid pMGD72 into pro-nuclei of C57BL/J6 x CBA F1 fertilized eggs.

To generate mice conditionally over-expressing a derivative of hPPARδ lacking the eleven carboxyterminal aminoacids residues (hPPARδΔAF2), the coding sequence of hPPARδ was amplified using primer PRMG15 (5´-CTAGTCTAGA**ATG**GAGCAGCCACAGGAGGAAGC- 3´) and PRMG16 (5´ -CTAGTCTAGATTAGTGCAGCGAGGTCTCGGTTTC-3´), (XbaI-sites underlined, ATG start codon in bold). This PCR product was cleaved with XbaI and cloned into plasmid pUHD10-3 (M. Gossen, unpublished, Genbank accession number U89931) creating pMGD10. The integrity of the insert ion was confirmed by sequencing and cleaved out using BamHI and ligated into pAHIR1-β-gal [132], digested with BglII resulting in plasmid pMGD18 (hPPARδΔAF2). The correct orientation was confirmed by sequencing. Four founder lines on a C57BL/6 background were generated for each transgene and

analysed for induction of transgene expression and suitable lines selected and brought forward for experimental analysis.

The expression of the transgene of choice is dependent on the activation of the mouse endogenous aryl hydrocarbon receptor (AhR). Activation is achieved by dietary administration of the AhR agonist (Indole-3-carbinol (I3C) - 0.25% (w/w)). Basal transcription from the *Cyp1a1* promoter in the absence of AhR agonist is very low, allowing for tight control of transgene expression.

All mice were fed *ad libitum*, and were kept under 12 hour light/dark cycles in humidity and temperature controlled environment. All procedures were done in accordance with regulations contained in the Animals and Scientific Procedures Act (1996) of the United Kingdom, and with the approval of the University of Dundee ethical committee.

2.2.2 Diets and administration of the compound

The animals were fed normal chow (standard RM1 laboratory animal feed SDS Ltd, Wickam, UK) or High Fat Diet (60% energy from fat, TestDiet, Richmond, USA).

GW501516 compound was administered to the mice in their diet. GW501516 was dissolved in DMSO and mixed with water, which was subsequently added to RM1 powder. The mass was formed into pellets, dried and used for experimental procedures with the final concentration of GW501516 0.0025% (w/w). The same method was used to incorporate I3C into the appropriate diets.

2.2.3 Measured parameters

Body weights were measured on a twice weekly basis and food intake was calculated weekly weigh-ins of the food pellets, before being refilled with fresh food.

2.3.4 Animals sacrifice and sample collection

At the termination of each experiment, animals were sacrificed using increasing concentration of CO_2. Blood was removed using cardiac puncture, followed by organ removal (liver, muscle (quadriceps), adipose tissue (visceral fat pad)). Collected tissues were snap frozen in liquid nitrogen and stored in -80ºC until further processing. Blood was collected in heparin coated tubes (Sarsted, Germany) and plasma separated by centrifugation and stored at -80ºC until further processing.

2.3 Determination of transgene detection.

Ear skin biopsy was taken from the animals which needed confirmation of presence of transgene expression.

2.3.1 DNA extraction

Skin biopsy specimens were suspended in 250µl of lysis buffer and 4µl of proteinase K (Qiagen) was added and whole mixture was incubated on heating block or in water bath at 55° overnight. After the incubation 365µl

Of chloroform was added along with 78.5 µl of NaCl (5M) and the samples were incubated on rotator at room temperature (RT) for 45 minutes. After this,

samples were centrifuged at 13000RPM for 10 min at room temperature. The upper aqueous phase was collected and added to 250 µl of isopropanol. Mixture was mixed by inversion and incubated at RT for 30 minutes. Samples were then centrifuged for 12 min at 13K RPM and the supernatant was discarded. Remaining pellet was washed with 70% ethanol and centrifuged again for 12 min at 13K RPM. Samples were then dried in the concentrator (Eppendorf) or dried under fume hood. Each pellet was re-suspended in TE buffer and stored in 4°C until needed.

2.3.2 Amplification of DNA

HotStarTaq Plus kit (Qiagen) was used to amplify DNA.

Reaction mixes contained 50-100ng of template DNA, 3 µl of Q solution 1.5 µl of Coral loading buffer, 0.6 µl of primers mix (from 10µM stock), 0.3 µl dNTPs mix (Promega), 0.12µl of DNA taq polymerase and the whole mixture was made up to 15µl with 8.28µl ddH_2O. The primers set used for detection of hPPARδ and hPPARδΔAF2 transgene were as follows: PRMG159 (5′-CCA ACC ACC CTG TCC CAG CTT G-3′) and PRMG160 (5′-ACA AAC TCT GCC CTG CTC TAT G-3′). The primers set used for detection of PPARδ-KO homozygotes were as follows: wild type allele: PBX9 (5′-AGA CAA TGA TGG TGT GCT CA-3′) and AB029 (5′-CTT TGG GGT GAG ACA GAC TGC GCA -3′). Mutant allele: PBX10 (5′-GCA GCT GCT CAG CTG CCT GC-3′) and UMS1 (5′-GCT CCT GAA GTC CAC AAT TCA CAG TCC -3′). The cycling parameters were as follows: 95°C for 5 min then 39 cycles of 94°C for 30 seconds, 60°C for 30 seconds, 72°C for 2 min and 72° for 10 min.

2.3.3 Agarose gel electrophoresis

DNA was routinely examined for the presence of the transgene by electrophoresis in 0.8% (w/v) agarose gel that contained TAE buffer with 0.5μg/ml ethidium bromide. The electrophoresis was performed at 120V for 48 min in TAE buffer. The 100bp or 1kb (New England Biolabs) markers were routinely run alongside analysis samples. DNA was visualised by UV illumination at 302nm.

2.4 Hepatic lipid analysis

2.4.1 Extraction of liver lipids

Total lipids from liver were extracted using Folch method [133]. 100mg of liver tissue was placed in 1.5ml eppendorf tube. 633μl of methanol was added and the tissue was homogenized using Polytron PT2100 homogenizer (Kinematica Luzern, Switzerland). After the dispersion, 1266μl of chloroform was add to get the final volume 20 times the volume of tissue sample (chloroform : methanol (2:1)). The mixture was agitated for 2 hours in an orbital shaker at room temperature. After the incubation, 0.2 volume of methanol (for 100mg of tissue 400μl of methanol) was added to lower the gravity of the mixture and the sample was centrifuged at 3000 RPM for 5 min. Supernatant was collected and placed in a clean 15ml centrifuge tube. Volume of chloroform was then added to restore 2:1 proportions of chloroform/methanol (e.g. for 100mg of tissue 800μl of chloroform). Mixture was vortexed for several seconds. Exact volume of the mixture was determined and 0.2 volume of 0.9% NaCl solution was added. Mixture was shaken on vortex for several seconds and centrifuged at 2000rpm for 20 minutes. After the centrifugation upper phase was removed and

discarded. The bottom organic phase was transferred in the 2 ml eppendorf tubes and evaporated under nitrogen stream at 60°C. The lipids were then either analyzed through Liquid chromatography mass spectrometry or re-suspended in lipid buffer and stored -80ºC until further analysis.

2.4.2 Liquid chromatography mass spectrometry analysis (LC MS) of phosphatidylcholine (GPC)

LC MS analysis of the lipid specimens extracted from the liver was carried out by Dr Jeffrey Huang (Biomarker and Drug Analysis Core Facility, University of Dundee, Ninewells Hospital) as follows: LC-MS analysis of GPC was performed on a Thermo Finnigan TSQ Quantum Ultra triplequadrupole mass spectrometer (ThermoFinnigan, San Jose, CA) in conjunction with a Thermo Dionex Ultimate 3000 LC and an electrospray source. The system was tuned using GPC (16:0/18:1)(Aventi). Five microliters of lipid extracts was injected to a Phenominex C18 column (50 x 2.1 mm). A 6 minute binary LC gradient elution from 50:50 CH_3CN/H_2O (solution A) to 90:10 isopropanol/CH_3CN (solution B) was employed. Both mobile phase solutions contained 0.1% formic acid. The lipids species containing phosphocholine were analyzed in a precursor ion scan (positive ion mode, mass range=740-820) monitoring neutral loss of 184.1, which corresponds to phosphocholine. The relative abundance of m/z 760 (representing 16:0/18:1-GPC) to m/z 758 (representing 16:0/18:2-GPC) was calculated.

2.4.3 Total cholesterol, TG, HDL and FFA biochemical assay.

Analysis of plasma and liver lipids was performed using RX Daytona clinical analyser (Randox, UK) with accordance to manufacturer's instructions.

2.5 Gene expression analysis

2.5.1 Preparation of cDNA from animal tissues.

Total RNA from the liver and muscle was prepared using the RNeasy or RNeasy Mini Fibrous tissue or RNeasy Lipid tissue Mini kit (Qiagen) following manufacturer instructions (Qiagen, UK). The procedure was performed also using automated extraction system Qiaqube (Qiagen, UK). During the extraction DNase treatment was performed to remove any possible traces of genomic DNA. To examine the integrity of RNA, sample of the RNA extracts was denaturated at 55°C for 5min and then run on agarose gel electrophoresis. When the bands were clearly visible, the RNA concentration of the samples were determined by NanoDrop 8000 (Thermo scientific, UK), and 500ng/sample of RNA was reverse transcribed using the High Capacity cDNA Reverse Transcription Kit following manufacturer instructions (Applied Biosystems).

2.5.2 Real-time PCR assay design

Taqman probes and primers were previously described [134] or were designed using Primer express 3.0 and were purchased from Applied Biosystems (UK) or from Eurofins MWG Operon(London, UK) or from Sigma/Aldrich (Gillingham,

Dorset, UK). Coding gene sequences were obtained from Genebank. Primer and probe sequences are shown in Table 1.

The primer sets flank the amplicon region and define the end points of the DNA probe labelled with FAM/TAMRA reporter and quencher dyes, respectively. The probe hybridizes with the amplicon and during PCR the 5´-3´ nuclease activity of taq polymerase cleaves the probe thereby separating the reporter and quencher, which results in fluorescence. Fluorescence is proportional to the quantity of cleaved probes and therefore to the amount of amplicons.

Table 2.1 Sequences of oligonucleotide primers and probes used in Taqman real time PCR. All sequences are shown in 5'→3' order.

Gene	Forward primer sequence 5'→3'	Probe sequence [FAM-Tamra]5'→3'	Reverse primer sequence 5'→3'
mHMGCoAs	TGGTGGATGGGAAGCTGTCTA	CCAAGGCCCGCAGGTAGCACTG	TTCTTGCGGTAGGCTGCATAG
mApoc3	CCAAGACGGTCCAGGATGC	CCATCCAGCCCCTGGCCACC	ACTTGCTCCAGTAGCCTTTCAGG
mCPT1	GGCTTAGTCGGGAGGCTCTG	AATCAACTCCTGGAAGAAACGCCTTATTCGAA	ACCCCTAAGGATGCCATTCTTG
mFAS	GGCATCATTGGGCACTCCTT	CCATCTGCATAGCCACAGGCAACCTC	GCTGCAAGCACAGCCTCTCT
mADRP	CAGCCAACGTCCGAGATTG	TGCCAGTGCCAGAGGTGCCGT	CACATCCTTCGCCCCAGT
mPPARα	GCGCAGCTCGTACAGGTCA	CAAGAAGACCGAGTCCGACGCAGC	TCTCTTGCAACAGTGGGTGC
mAcox1	TGACCGTTAAGGTCTTTGCAGA	AACTCCCCAAGATTCAAGACAGAGCCGT	AGGTTCCTCAGCACGGCTT
mPPARγ	CTGACCCAATGGTTGCTGATTAC	AAATATGACCTGAAGCTCCAAGAATACCAAAGTG	TGGAGATGCAGGTTCTACTTTGA
mPPARδ	GACCAGAACACACGTTTCCTTC	AGCAGCTGTGCAGACCTCTCCCAGA	CCATCACAGCCCATCTGCA
hPPARδ	GGGACCACAGCATGCACTTC	CCAGCAGCTACACAGACCTCTCCCGG	TGCAGTTGGTCCAGCAGTGA
mCD36	TGAAAAGTCTCGGACATTGAGATTC	TTTCCTCTGACATTTGC	AGATCCGAACACAGCGTAGATAGA
mUCP1	GCAGATATCATCACCTTCCCG	TGGACACTGCCAAAGTCCGCCTTC	CCTGGCCTTCACCTTGGAT
mUCP2	TCCTGAAAGCCAACCT	AGATGACCTCCCTTGCCACTTCAC	CGATGACGGTGGTGCA

	CATGA	TTCTG	GA
mUCP3	GAAGATGGTGGCTCAGGAGG	CCACGGCCTTCTACAAAGGATTTGTGC	AAGCTCCCAGACGCAGAAAG
mPDK4	GGAAGTATCGACCCAAACTGTGA	CACTCAAAGGCATCTTGGACTACTGCTACCA	GGTCGCAGAGCATCTTTGC
mANGPTL4	GCTTTGCATCCTGGGACGAG	ACTTGCTGGCTCACGGGCTGCTAC	CCCTGACAAGCGTTACCACAG
mCDKN2C	GCGCTGCAGGTTATGAAAC	CCTGGCAATCTCCGGATTTCCA	TTAGCACCTCTGAGGAGAAGC
mGAPDH	GCCAAGGTCATCCATGACAAC	CTCATGACCACAGTCCATGCCA	GGGGCCATCCACAGTCTTC

2.5.3 Taqman quantitative Real Time PCR

The cDNA was initially diluted 1:5 and optionally further dilutions were made on the basis of pilot assays which were run with single samples for each tissue to determine optimal dilution of the cDNA for each gene assay. The cDNA samples were collected into single 96 well plate and then scaled up to 384 well daughter plates format using robot (FluidX xpp-721) (resulting in 4 copies per each sample). A premix consisting of 3µl of 5x Taqman Master mix (Applied Biosystems, UK), 0.5µl forward primer (from 18µM stock), 0.5µl reverse primer (from 18µM stock), 0.4µl Taqman fluorogenic probe (from 5µM stock) and 1.6µl of water was made. Then a robotic system (Equator, Deerac Fluidics) was used to add 6µl of premix to 4 µl of cDNA into the 384 well plates making up 10µl total volume of mixture/well. The ABI Prism 7900 sequence detector (Applied Biosystems) was used to perform RT-PCR reaction. Cycling parameters were as follows: 50°C for 2 minutes, 95°C for 10 minutes, 40 cycles of 95°C for 15 seconds, 60°C for 1 minute. Data was acquired and processed with Sequence Detector 1.6.3 software (Applied Biosystems).

2.5.4 Gene expression normalization

The test genes expression levels were normalized to remove possible differences such as RNA quality and pipetting errors that could distort the results. 18s RNA or mouse glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were used as housekeeping genes. A following formula was used for normalizing:

$$2^{((\text{target gene Ct value} - \text{housekeeping gene Ct value}) \times (-1))}$$

Where: Ct value = threshold cycle value for each sample (where fluorescence signal was recorded as statistically significant above background levels.

2.6 Microarray analysis

2.6.1 Samples processing

Total RNA was harvested from liver samples using the RNeasy kit (Qiagen) according to the manufacturer's instructions, including a DNase digestion step. Total RNA was quantified on a NanoDrop spectrophotometer (ND-8000) and samples were amplified and labelled using Illumina TotalPrep RNA amplification Kit (Invitrogen, UK). From this point samples were processed until raw data results in Wellcome Trust Clinical Research Facility, Western General Hospital, Edinburgh by Ms Louise Evenden. All samples passed quality check using Agilent 2100 Bioanalyser and 1.5ug/biotin-labelled sample was hybridized with MouseWG-6 v2.0 Expression BeadChips (Illumina,USA). BeadChips were scanned using an Illumina BeadArray reader and raw data were acquired using GenomeStudio software. MouseWG-6 v2.0 Expression BeadChip contains 45,281 transcripts that can be analyzed in one sample. The beadchip contains

the probes from the MouseRef-8 beadchip with additional probe design based on RIKEN FANTOM 2 database and other data sources.

2.6.2 Analysis of the microarray data

Expression data were normalized (average normalization) in GenomeStudio with background extraction. Microarray data were analyzed using GenomeStudio, MeV v 4.8.1 and MS Excel. False discovery rate method (FDR) [135] was used to correct for multiple testing effects. Differentially expressed genes were identified by determining the fold change and P values of the t-test. Genes whose expression intensity changed by >1.75 fold up or down with $P<0.05$ and $FDR<0.05$ were considered to exhibit differential expression in the microarray experiments. Correlation tests were considered to be significant if correlation $P<0.05$ and $FDR<0.05$ was obtained.

2.7. Histological analysis of liver tissue

2.7.1 Preparation of tissues for sectioning

Frozen liver tissue was placed into bijous containing fix which had been cooled down to 4°C. Liver was fixed in 1% PFA/PBS/2mM MgCl2 for 3 hours at 4°C. Then tissues were washed 3 times with PBS containing 2mM $MgCl_2$. Tissues were then dehydrated overnight at 4°C in PBS/2mM $MgCl_2$ containing 30% sucrose. After dehydration, tissues were embedded in Optimal Cutting Temperature compound (OCT), mounted on the plastic moulds and placed on the dry ice/isopentane bath for 5-10 minutes. Moulds with tissues were stored in -80°C or continued with staining protocol.

2.7.2 Oil red O staining

The histological mechanism of the staining of lipids is invariably a function of the physical properties of the dye being more soluble in the lipid to be demonstrated than in the vehicular solvent. The polyazo group of dyes include the oil red series, the Sudan red series, and the Sudan blacks. For negative reference one section was treated with a 50:50 acetone/xylene mixture for 5 minutes, wash in water and continued with the protocol. Frozen sections were cut (10 microns) on cryostat and mounted on Optiplus positively charged microscope slides and air-dried to the slides for 30 minutes only. Then slides were immersed into the formalin and fixed at 4°C for 30-45 minutes. After this, slides were washed with running tap water for 7 minutes (or until OCT is completely disappeared from the slide) and they were rinse in distilled water. Subsequently they were placed in 60% isopropanol for 1 minute only, to remove water and then placed in Oil Red O (ORO) working solution for 15 minutes. ORO working solution was made as follows: 0.5g ORO powder was mixed with 100ml of isopropanol. Then 30 ml of the stock stain was mixed with 20 ml of 1% water dextrin solution, allowed to stand for 30 minutes, and filter through 0.2µm filter. After incubation with ORO slides were rinsed with 60% isopropanol and placed in clean 60% isopropanol for 2 minutes only. After that they were rinsed in distilled water and stained with Mayer's hematoxylin for 30 sec. After this step slides were rinsed in distilled water and placed in running tap water for 10-12 minutes for "bluing". They were rinsed in distilled water and mounted with cover glass in glycerine jelly. Sections were viewed under a light microscope.

2.8 Statistical analysis

GraphPad Prism 5.0 (Graphpad Software, Inc, CA, USA), MS Excel, GenomeStudio (Illumina) and MeV v 4.8.1 were used for statistical analysis. Two-way ANOVA with Bonferroni post tests, student t test and correlation was used to calculate statistical significance. $P<0.05$ and $FDR<0.05$ for multiple testing were considered as significant.

2.9 Companies from which chemicals and kits were obtained

Agilent technologies (Stockport, Cheshire, UK)

Anachem (Bedfordshire, UK)

Avanti Polar Lipids (Alabaster, Alabma, USA)

Bio-Rad laboratories (Hemel Hempstead, Herts, UK)

Charles River Ltd (Margate, Kent, UK)

Fluka (Pool, Dorset, UK)

GraphPad Inc. (San Diego, USA)

Invitrogen (Paisley, Scotland)

MWG Biotech (Ebersberg, Germany)

New England Biolabs (Hitchin, Herdfordshire, UK)

Nunc Brand products (Roskilde, Denmark)

Applied Biosystems (Foster City California USA)

Promega UK (Southampton, Hampshire, UK)

Randox (Crumlin, Antrim, United Kingdom)

Qiagen Ltd (Crawley, UK)

Sigma-Aldrich (Poole, Dorset, UK)

VWR (Poole, Dorset, UK)

Chapter 3 Anti-obesity and metabolic properties of PPARδ agonism, in non-transgenic mice.

3.1 Introduction

Although it is generally accepted that PPARδ is expressed ubiquitously [136], some data indicate that its expression level in mouse skeletal muscle is relatively low when compared to liver, small intestine or skin [137]. Despite that fact, important functions have been assigned to PPARδ action in muscle [121,123,138]. In liver however, aspects of PPARδ activation are less understood. The situation becomes even more complicated because of the fact that there is a variety of synthetic PPAR δ ligands available, which have been tested in various animal models and men. Having different potency and selectivity towards PPARδ, it is not surprising they can produce different results in diverse model organisms [139]. The aim of the following study was to show the effect of the PPARδ activation by one potent and up to date synthetic agonist available – GW501516 in wild type C57BL mice. GW501516 is more than 1000 times more selective for PPARδ than for the rest of the PPAR family members (EC50=0.001μM for PPARδ and EC50=10μM for PPARα and PPARγ by luciferase reporter gene transactivation assay in COS7 cells) [127].

So far GW501516 use in animal models produced variety of results. In general it is accepted that in rodents GW501516 causes weight loss or prevents body mass gain [81,122,140]. The other aspects of GW501516 treatment vary and may be dependent on experimental design, animal model used etc. Whereas some studies shown promotion of steatosis in rodent liver after GW501516 treatment [124] others suggest it even as a drug candidate to treat NASH [129,141]. This issue needs to be clarified as long as PPARδ synthetic

ligands are considered to be use therapeutically in humans for treating aspects of metabolic syndrome. This chapter aims to assess the short and long-term effect of PPARδ ligand administration on body mass, hepatic and plasma lipid profiles and gene expression in wild type mice.

3.2 Results

30 female C57BL/6 background mice were divided into two branches: one fed standard chow (RMI diet) and the second - chow supplemented with 0.005% of GW501515 (7mg/kg/day). 10 animals (5 per each group) were sacrificed at 2, 4 and 8 weeks time. Mice were 10 weeks old at the beginning of experiment. Before the sacrifice, animals were fasted to ensure adequate plasma lipids measurements.

Throughout the duration of experiment, treated animals showed no sign of obvious ill health. After introduction of GW501516 into the diet, treated mice started to lose weight in the first 2 weeks of experiment. After that time point, the weight loss trend reversed and weight gain was seen until the end of the experiment (Figure 3.1A). Control mice were gaining weight steadily throughout the whole 8 weeks (Figure 3.1A). After 4 weeks difference in weight gain between control and treated group was 10.3 fold (Figure 3.1B). And the weight difference in terminal weights reached 2.6 fold (Figure 3.1B). Thus influence of PPARδ ligand on weight gain after 4 and 8 weeks was considered as very statistically significant ($P<0.01$).

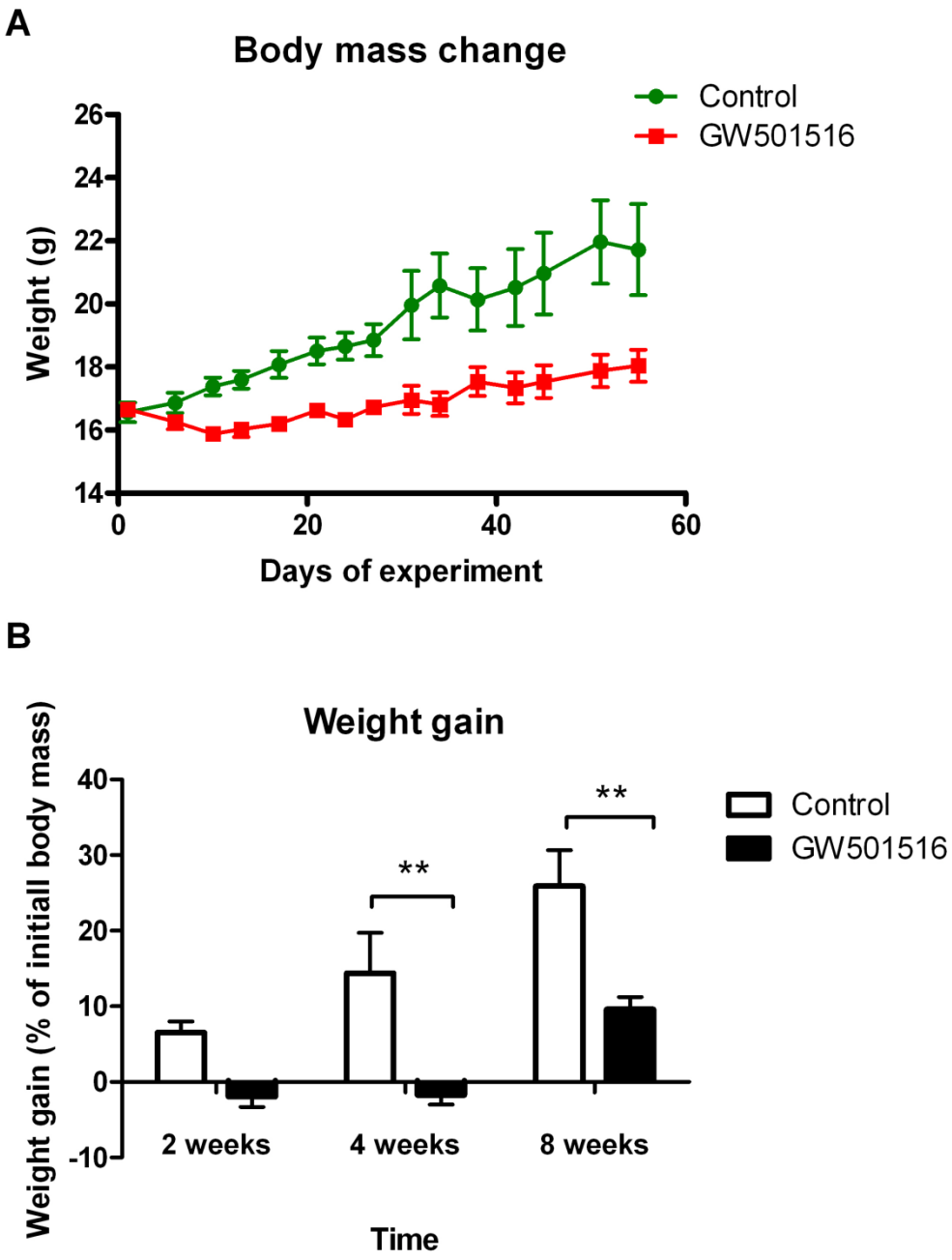

Figure 3.1 GW501516 treatment prevents diet induced obesity. (A) Body mass change throughout 8 weeks of feeding with PPARδ agonist. (B) Percentage weight difference between control animals and fed GW501516-supplemented diet. Significance is indicated as (** $p<0.01$; *** $p<0.001$), n=5mice/group. Data are expressed as means ± SEM.

In parallel experiment, with exactly the same experimental design, but without fasting the mice, results show the same trend of weight loss (data not shown). Additionally, food consumption was monitored. Although changing over time, an average food intake throughout the whole experiment in animals fed diet supplemented with PPARδ ligand was lower than in the control group fed plain chow (Figure 3.2). GW501516 at dose of 7mg/kg/day (0.005%; w/w) has led to reduction in food consumption by 8%. This decrease in appetite was statistically significant (p=0.0322).

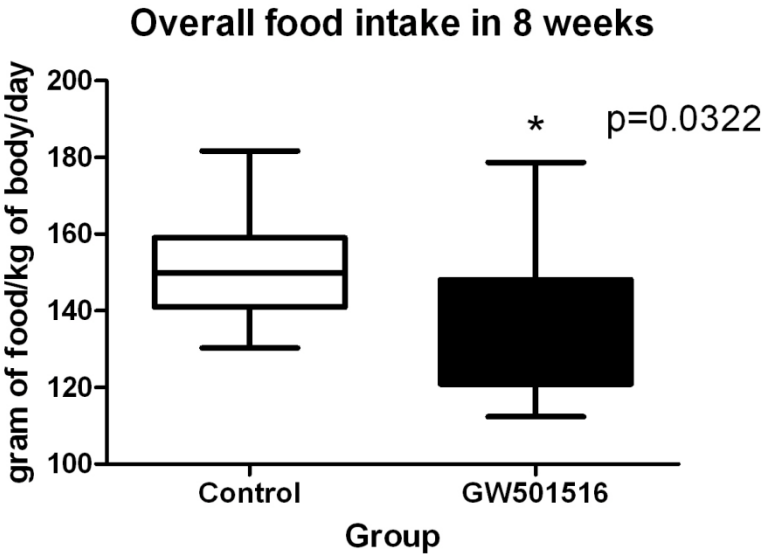

Figure 3.2 Food intake in animals fed normal chow and diet supplemented in GW501516. n=15mice/group. Data are expressed as means ± SEM.

Plasma levels of GW501516 concentration in treated animals was found to be at the level of 1μmol/L, with no measurable amount detected in animals

fed control diet (Figure 3.3 A). Blood levels of triglyceride were significantly lower in mice fed diet supplemented with PPARδ ligand at every measured time point (Figure 3.3 B). Although GW501516 treatment significantly increased, the levels of plasma total cholesterol (at 4 and 8 weeks time; Figure 3.3 C), the High Density Lipoprotein (HDL) levels showed the same trend (Figure 3.3 D).

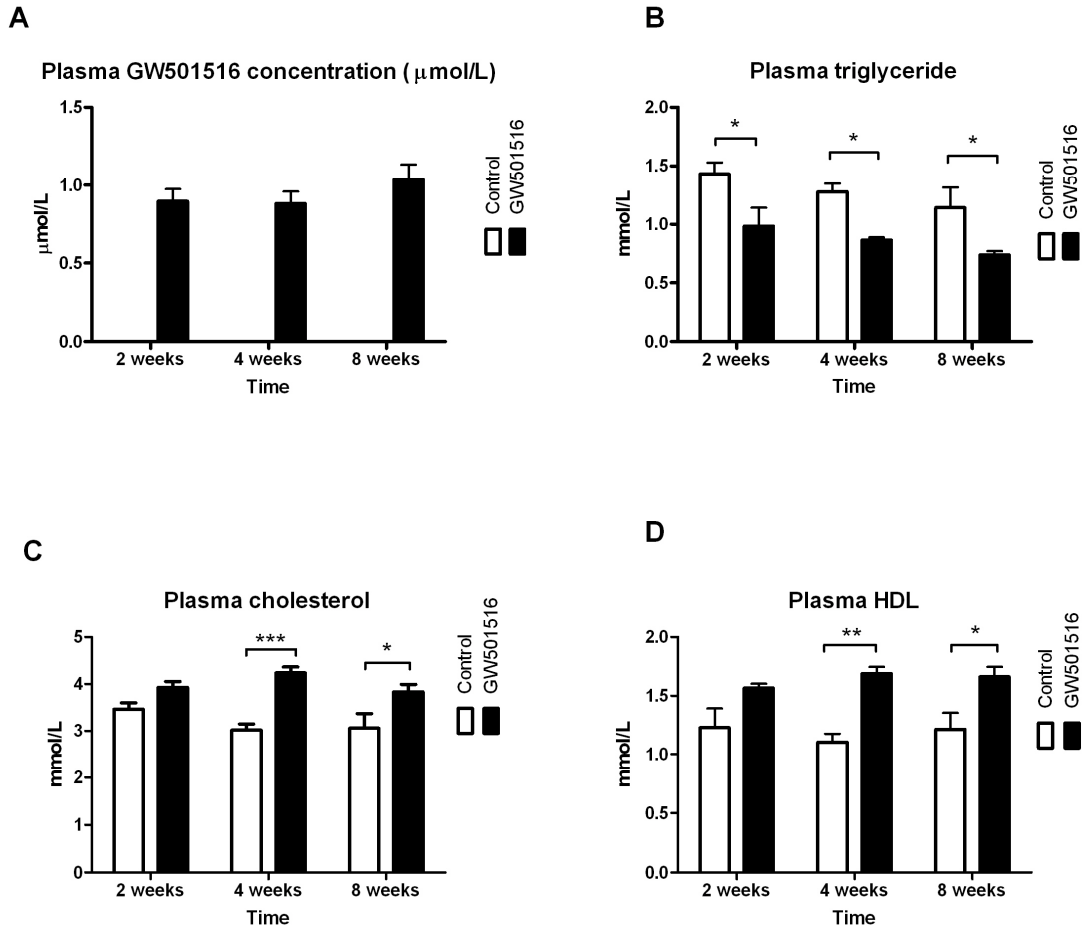

Figure 3.3. Effects of PPARδ agonism on the serum lipid profile of C57/BL6 mice. (A) Plasma GW501516 concentration. (B) Improvement in blood triglyceride profiles in treated animals. (C) Increase in total plasma cholesterol and (D) HDL levels. Significance is indicated as (*$p<0.05$; ** $p<0.01$; *** $p<0.001$), n=5mice/group. Data are expressed as means ± SEM.

Plasma Free Fatty Acids (FFA) and glucose plasma levels showed no significant differences between groups at any time point (data no shown). Plasma glucose levels were significantly lower after 4 weeks ($P<0.05$; data not shown), with no difference between groups after 2 and 8 weeks time.

In the plasma derived from non-fasted animals, insulin levels had no significant differences between groups or throughout the experiment duration (Figure 3.4A). Another hormone levels, leptin, had tendency to be at lower level in treated groups, when compared to controls. However, only differences at 2 and 8 week time points were significant ($P<0.05$; Figure 3.4B).

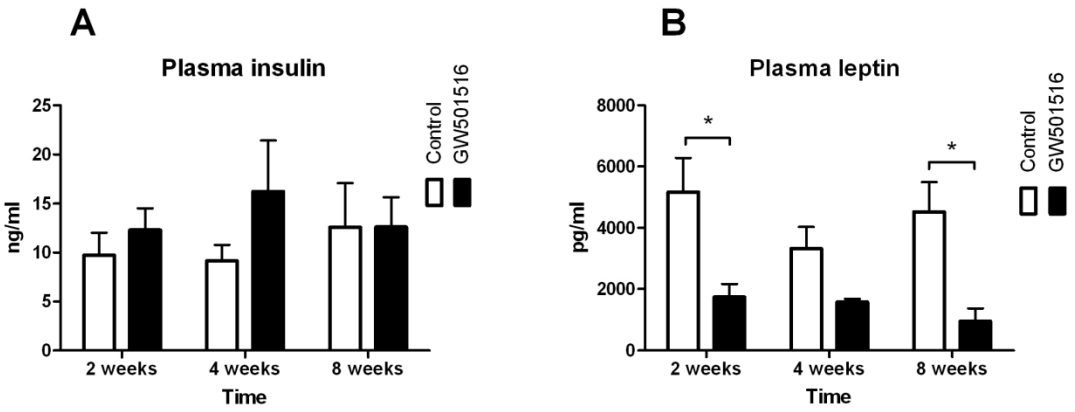

Figure 3.4. Plasma insulin and leptin levels in non-fasted animals fed control diet or diet supplemented with 0.005% GW501516 – a PPARδ agonist. n=5 mice/group. Data are expressed as means ± SEM.

After 2 weeks, levels of hepatic fat (triglyceride) showed no variation between groups. However, after 4 weeks, mice fed diet supplemented with PPARδ ligand had accumulated 70% more triglyceride than control group ($P<0.01$; Figure 3.5). At the end of the experiment though, the fatty liver phenotype was reversed, with control group having 70% more intrahepatic fat

when compared to treated group (P<0.001). Level of liver triglyceride after 8 weeks in treated group was comparable to level found in the same group after 2 weeks. When comparing 4 and 8 weeks time points, hepatic fat level within treated groups was down by 62% at the end of the experiment (P<0.001; Figure 3.5). Level of liver cholesterol showed no changes between groups or throughout the duration of experiment (data not shown).

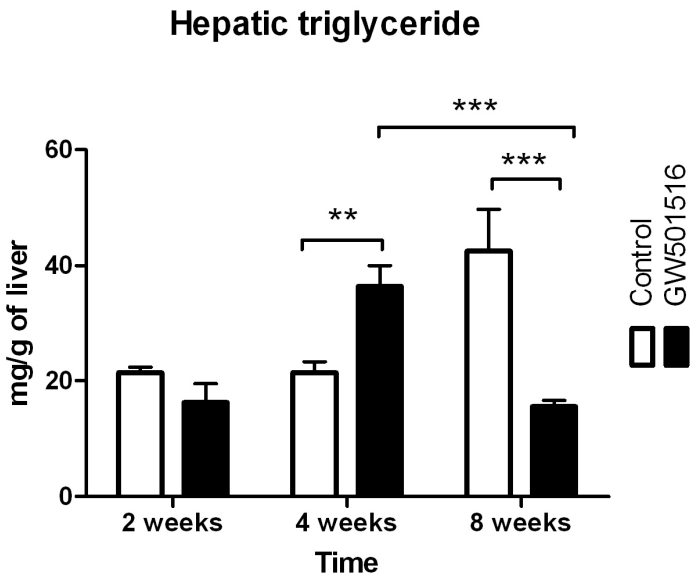

Figure 3.5 Levels of hepatic triglyceride in non-transgenic mice fed diet supplemented with PPARδ ligand. 4 weeks treatment increases the hepatic triglyceride content (P<0.01), whereas 8 weeks was protective against GW501516-induced liver steatosis in non-transgenic mice (P<0.001). n=5 mice/group. Data are expressed as means ± SEM.

Oil red O staining of frozen liver sections revealed and confirmed deposition of the triglyceride inside hepatocytes. The size and the manner of infiltration of triglyceride droplets indicate microvescicular rather than macrovescicular type of fat deposition in livers of mice fed diet supplemented with GW501516.

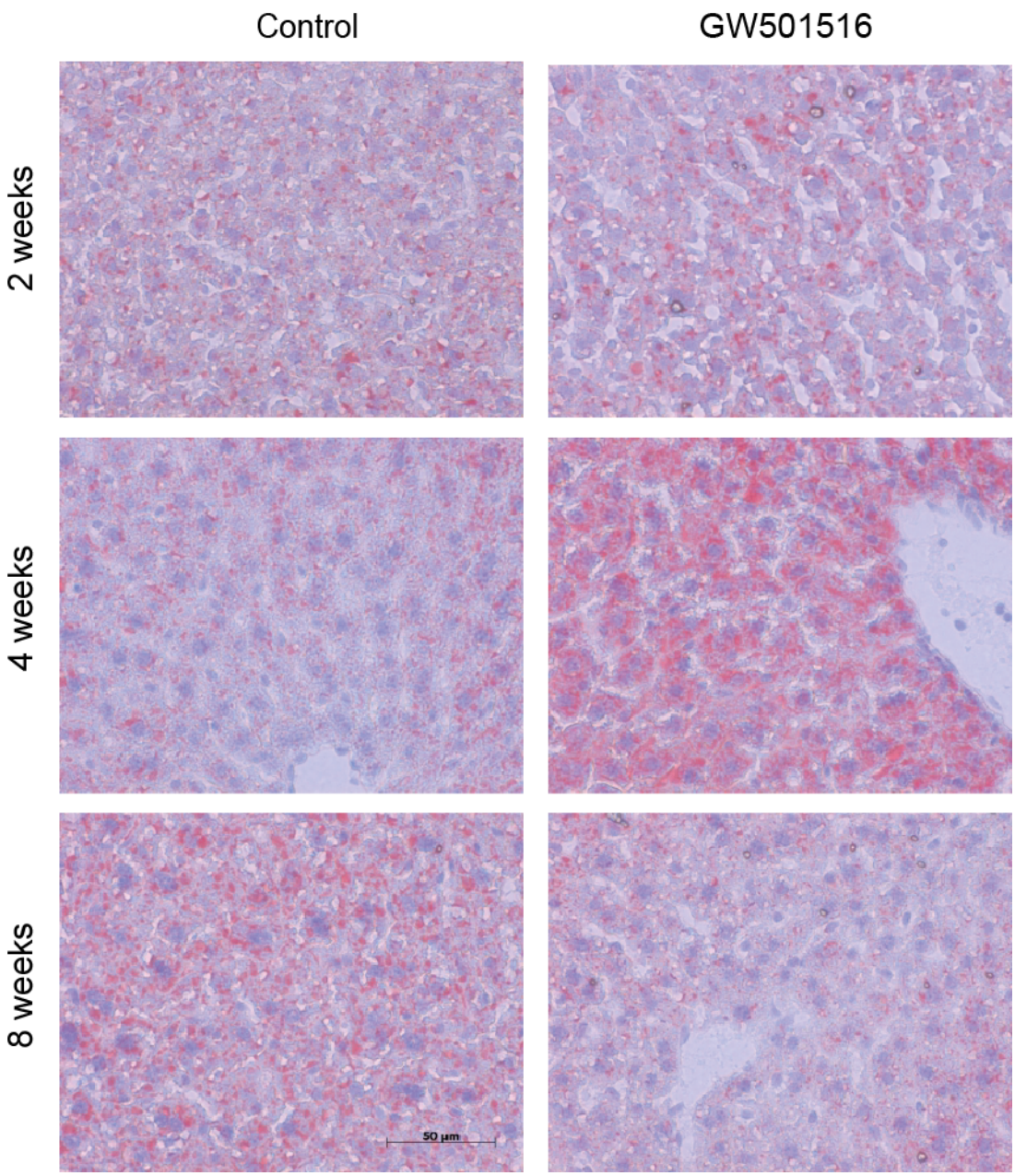

Figure 3.6 Oil Red O staining of liver sections. Each picture represents typical example of hepatic fat deposition in separate group. Magnification: 400X, scale bar = 50µm.

Gene expression analysis revealed that mRNA expression of the three PPAR isoforms in liver and muscle showed no variability between groups and throughout the length of experiment with the exception of PPARδ mRNA expression in muscle (Figure 3.7).

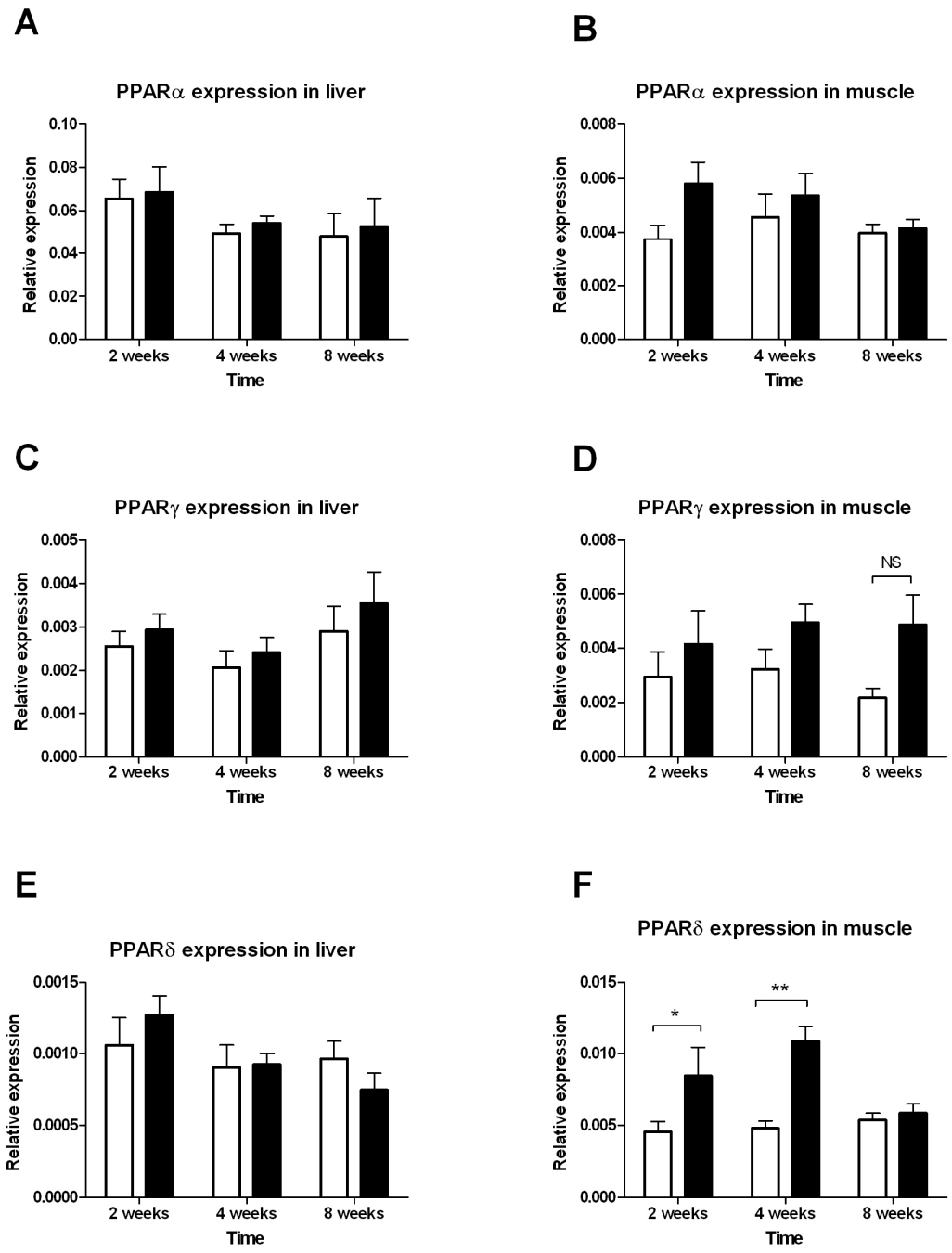

Figure 3.7. Expression of messenger RNA of PPAR receptors in liver and muscle. Only PPARδ expression in muscle showed difference after GW501516 treatment after 2 and 4 weeks time ($P<0.05$ and $P<0.01$ respectively). Samples derived from fasting animals. White bars – mice fed control diet, black bars - mice fed diet supplemented with GW501516. n=5 mice/group. Data are expressed as means ± SEM.

Bearing in mind that PPARδ transcriptional activity targets genes which participate in fatty acid processing and utilization, mRNA expression of several marker genes was measured. Adipophilin (Adipose Differentation-Related Protein: ADRP) a protein that coats intracellular lipid droplets is a marker of triglyceride accumulation, and direct downstream target of PPARδ receptor. Although the presence of intrahepatic fat was clearly shown before the real time, surprisingly, PCR did not reveal major changes throughout time of the experiment and between groups (Fig 3.8A). Apolipoprotein C3 (Apoc3), an inhibitor of lipoprotein lipase and thus marker of hypertriglyceridemia was down-regulated in livers of GW501516 treated animals when compared to controls. The trend was visible after each time point, although only after 4 (down by 32%) and 8 weeks time (down by 29%) difference turned out to be significant ($P<0.05$ and $P<0.01$ respectively) (3.8B). Change in this gene expression levels is consistent with lower plasma triglyceride levels found in mice treated with GW501516 (Fig 3.3A).

Considering the fact that the plasma cholesterols levels were higher in PPARδ agonist treated groups, HMG-CoA synthase (HMG-CoAS), which catalyzes the reaction to form 3-hydroxy-3-methylglutaryl-CoA and therefore is important enzyme in cholesterol synthesis pathway was chosen for the marker gene. Enzyme's mRNA was up-regulated in treated group after 4 weeks (by 68%; $P<0.01$) and at 8 weeks time. However, this was not statistically significant at the final time point (Fig 3.8 C).

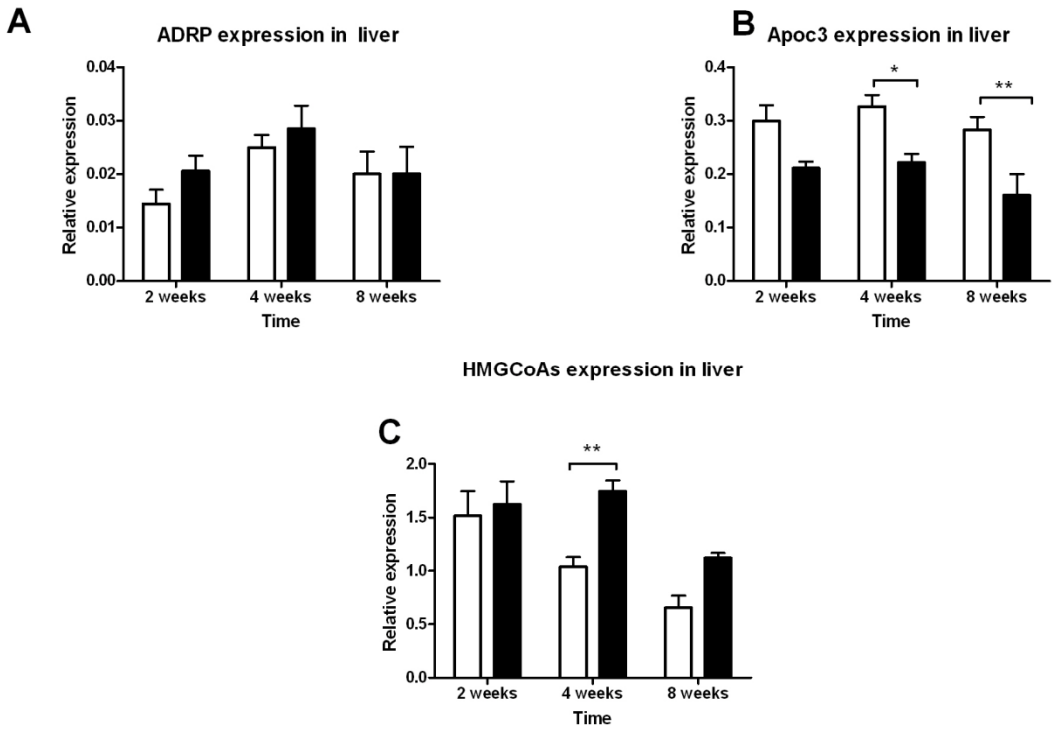

Figure 3.8 Levels of hepatic mRNA of Adipophilin (ADRP) with no treatment or time related changes (A); Apolipoprotein C3 (Apoc3) with tendency to drop in GW501516 treated mice (B) and HMG-CoA synthase (HMG-CoAS) (C), were up-regulation is consistent with plasma total cholesterol levels (Fig 3.3C). White bars – mice fed control diet, black bars - mice fed diet supplemented with GW501516. Two-way ANOVA, significance is indicated as (*$p<0.05$; ** $p<0.01$), n=5 mice/group. Data are expressed as means ± SEM.

Based on gene expression by real time PCR no evidence was found that presence of excessive liver triglyceride in PPARδ agonist treated group after 4 weeks was caused by fatty acid synthesis *de novo*. The level of hepatic PPARγ mRNA, a master of adipocyte differentiation and fatty acid anabolism, had not changed at any time point (Fig 3.7C). The level of mRNA of protein directly involved in fatty acid synthesis, Fatty acid synthase (FAS) in liver after 4 weeks was actually lower in treated group by 60%, when compared to untreated group

from the same period. However, after reduction of the lipid content in livers of treated animals at 8 weeks time (Fig 3.5) the level of FAS has risen by 333% when compared to the same group from 4 weeks time point (Fig 3.9A). In muscle, the level of FAS mRNA was found only significantly different after 8 weeks, where it was 3.7 fold higher in treated animals, when compared to controls (Fig 3.9B).

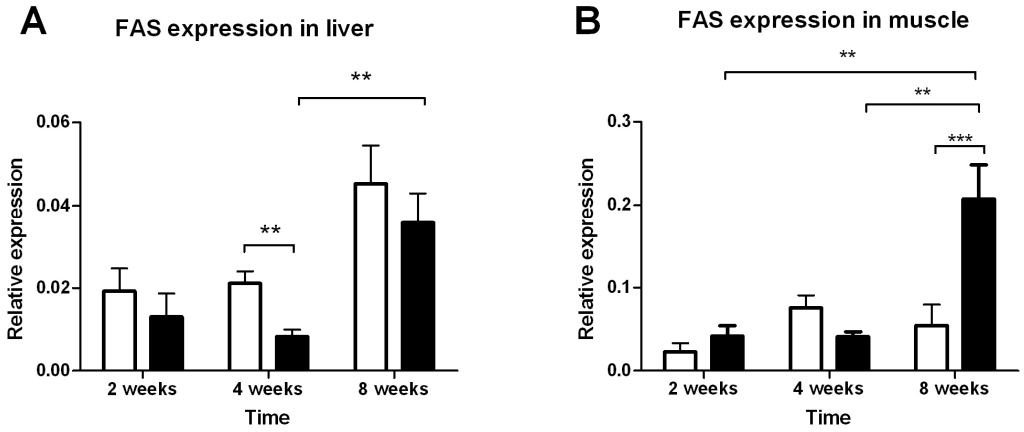

Figure 3.9. Level of Fatty acid synthase (FAS) in livers (A) and muscle (B) in animals fed control diet (white bars) and animals fed diet supplemented with PPARδ agonist. In both organs no positive correlation was found between increased triglyceride levels after 4 weeks and up-regulation of FAS. Test used: two-way ANOVA, significance is indicated as (** $p<0.01$; ***$p<0.001$), n=5 mice/group. Data are expressed as means ± SEM.

Although PPARδ agonism is generally responsible for induction of lipid catabolism, an impaired β-oxidation process could promote fat accumulation and as a result, liver steatosis. Several β-oxidation and fat catabolism marker genes were chosen for testing that hypothesis. Expression of peroxisomal acyl-coenzyme A oxidase 1 (Acox1), which is the first enzyme of the fatty acid beta-

oxidation pathway, was not changed substantially between groups or over the time of experiment (Fig 3.10A). On the other hand, mRNA levels of carnitine palmitoyltransferase I (CPT1), which mediates the transport of long chain fatty acids across the mitochondrial membrane, was increased 5 fold in steatotic livers (4 weeks, treated animals) (Fig 3.10B). Also hepatic expression of mRNA of uncoupling protein 2 (UCP2), which decreases reactive oxygen species production during lipid oxidation was increased at 2, 4 and 8 weeks time in GW501516 treated mice (by 65%, 74% and 163% respectively) (Fig 3.10C).

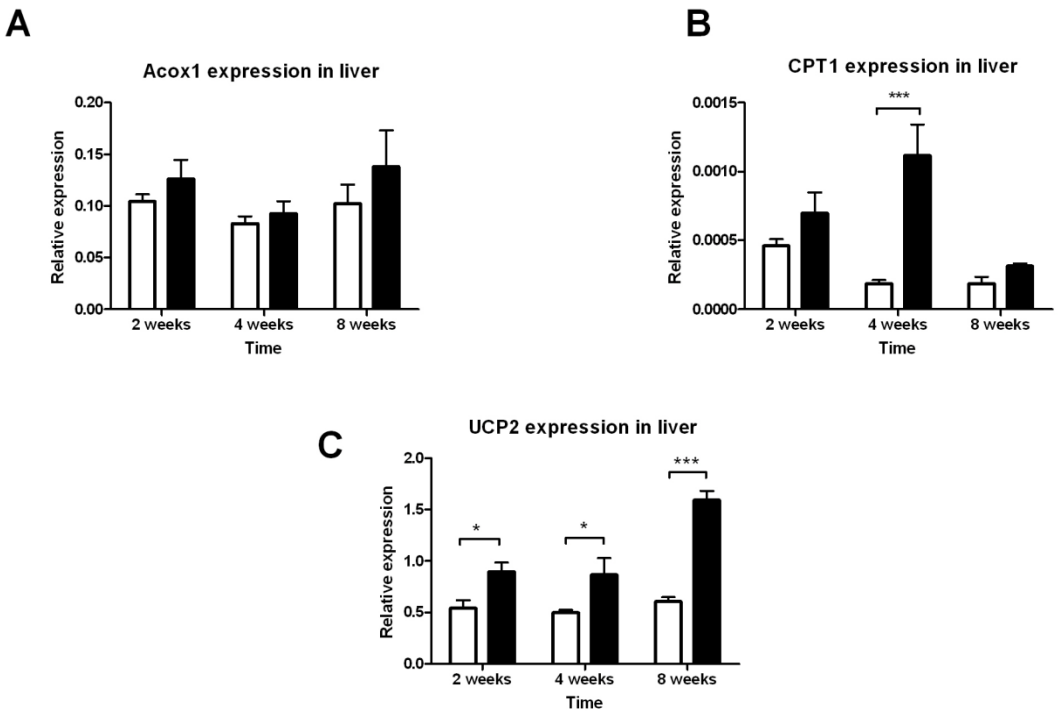

Figure 3.10. Examples of regulation of the genes involved in lipid oxidation upon PPARδ agonist treatment. Acox1 expression shows no changes in the livers of animals with increased fat content (4 weeks, treated group) (A). Hepatic CPT1 expression is up-regulated by PPARδ ligand in livers of treated animals (4 weeks) (B). UCP2 mRNA levels are elevated significantly at every time point by GW501516 treatment. White bars – mice fed control diet, black bars - mice fed diet supplemented with GW501516.

Test used: two-way ANOVA, significance is indicated as (*p<0.05; ***p<0.001), n=5 mice/group. Data are expressed as means ± SEM.

Using muscle-derived mRNA, more global expression pattern of lipid oxidation genes were examined. CPT1 expression after 4 weeks increased by 53% in treated group, when compared with control (Fig 3.11A). Uncoupling protein 3 (UCP3), which plays role in neutralization of protein oxidation (and therefore is usually correlated with lipid oxidation) was up-regulated in treated group after 2 and 4 weeks time points (by 143% and 123% respectively) (Fig 3.11B). Additionally, pyruvate dehydrogenase kinase isozyme 4 (PDK4), which inhibits carbohydrate metabolism in favour of lipid based metabolism in muscle, had similar pattern of expression in skeletal muscle as mentioned UCP3. Up-regulation of the isoenzyme was significantly different in treated groups at 2 (68%) and 4 (217%) weeks time (Fig 3.11C).

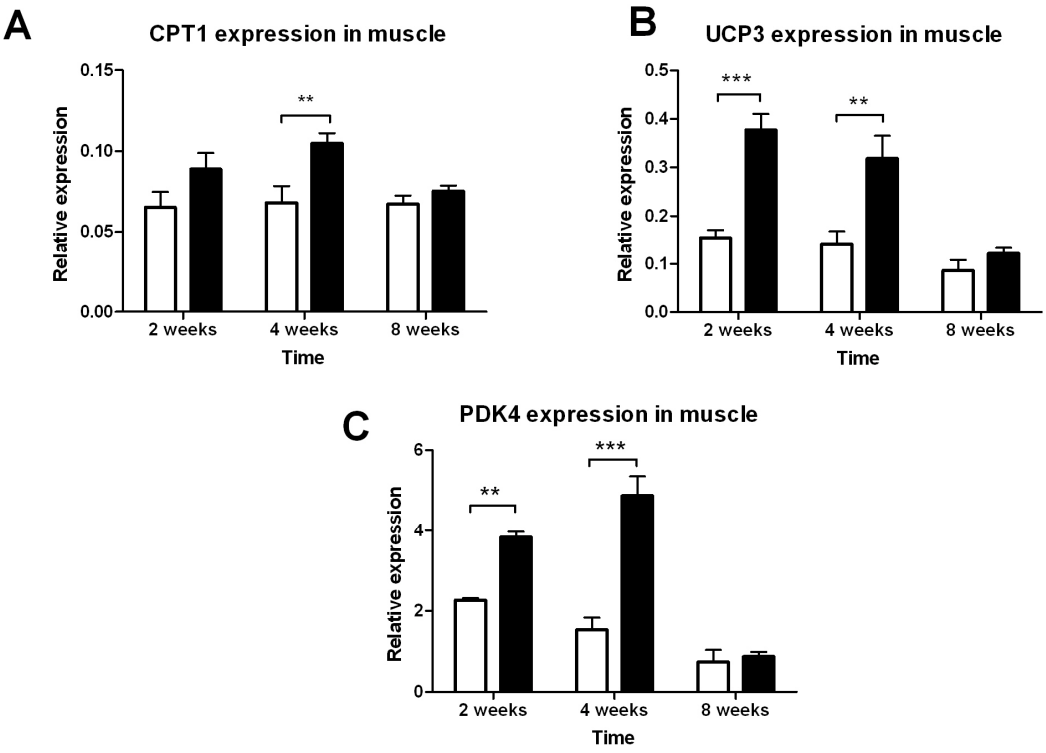

Figure 3.11 Up-regulation of the genes involved in fatty acid oxidation in muscle. CPT1 (A) mRNA levels were higher in treated animals after 4 weeks. UCP3 (B) and PDK4 (C) reached significantly higher levels after 2 and 4 weeks. White bars – mice fed control diet, black bars - mice fed diet supplemented with GW501516. Test used: two-way ANOVA, significance is indicated as (** $p<0.01$; ***$p<0.001$), n=5 mice/group. Data are expressed as means ± SEM.

3.3 Discussion

This chapter proceeded to investigate phenotypic and transcriptional aspects of using GW501516 as a selective PPARδ activator in non-transgenic mice. Consistent with many reports published before [121,142,143], activation of PPARδ led in this study to prevention of body mass increase and improvement of plasma lipid profile.

PPARδ role in modulation of appetite and plasma lipid profile

As the main function of this transcription factor is to switch from carbohydrate-based metabolism to lipid-based metabolism, activation of PPARδ enhances lipid catabolism in various tissues, thereby retarding weight gain. The reduced appetite of mice fed diet supplemented in GW501516 might contribute to overall weigh reduction found in these animals. Although it is difficult to say whether reduced food intake is a direct result of PPARδ activation or rather a side effect of pharmacologically-provoked lipid catabolism. A similar finding was discovered in study done by Perreault et al [144]. In their work, diet-induced obese and *ob/ob* mice were fed diet supplemented with a PPAR pan agonist (compound 4), which caused appetite suppression along with body weight loss. Although PPAR pan agonist activates all three subtypes of PPAR family,

authors associated mainly activation of PPARα as the main contributor to reduced food intake. In our study, however, the agonist used, GW501516 (as it was mentioned before in introduction to chapter 3) is more than 1,000-fold selective for PPARδ over the other subtypes of PPAR family [127], suggesting that PPARδ might also play role in this phenomenon.

As previously reported in human and primate studies [116,127], GW501516 treatment was also capable of reducing plasma triglyceride in mice. On the other hand, raised HDL levels were not observed in studies in men, whereas in primates and in our study in C57BL mice, this HDL raising is apparent. Although rise in HDL could be partially explained by increased concentration of total plasma cholesterol also found in our study, nevertheless, ability to modify HDL levels is still important feature of PPARδ agonism in the context of its metabolic action.

In this study, lack of influence of GW501516 on insulin levels was in contrast to its action on leptin levels, where PPARδ agonist was capable of lowering leptin significantly almost at every measured time point. Similar results was described in work done by Chen et al, where GW501516 was able to improve hyperleptinaemia in monosodium l-glutamate metabolic syndrome mice [122]. Generally accepted evidence suggests that leptin has a role within the central nervous system to modulate food intake and thermogenesis [62]. It is assumed that leptin concentrations are, in general, proportional to body fat mass. However, circulating leptin levels tend to drop sharply e.g. during fasting. Considering the fact that PPARδ is particularly active during states of exercise or restriction of energy intake, pharmacological activation of PPARδ could have similar effect as mentioned physiological conditions on this hormone levels.

Role of PPARδ agonists in liver lipid metabolism in wild-type mice

Several of the previous studies have suggested some role of synthetic PPARδ agonists in promoting hepatic lipid accumulation [124,128], whereas other considered it neutral or even negative [116,130]. In our work, we show evidence that in non-transgenic mice treated with GW501516 there was time dependent liver fat fluctuation. Animals fed control diet steadily gained body mass, which eventually after 8 weeks resulted in increased hepatic lipids. In contrast, the mice fed diet supplemented with GW501516 after 4 weeks had significantly higher liver triglyceride content, while still not gaining any body mass. However, at the end of the experiment, treated group had less triglyceride in the liver than control group. It is possible that PPARα subtype might be responsible for clearance of the hepatic lipids in the long term. Although GW501516 selectivity for PPARδ was quoted before, PPARα to PPARδ proportions in rodent liver are well in favour for the former. Non-selective activation of PPARα by GW501516 accumulating in the liver or by PPARδ downstream activity on PPARα could be possible explanation of hepatic triglyceride fluctuation, in spite of stable GW501516 blood plasma's concentrations.

Lee et al [124] proposed promotion of fatty acid synthesis as an explanation for observed fat accumulation in livers of mice fed GW501516 to allow the utilisation of glucose and providing fuel for fat burning in muscle. However, in contrast to their finding, we show that in our study, fatty acid synthase (FAS) was not induced by GW501516 and was not positively

correlated with level of hepatic triglyceride. Therefore with this evidence it is difficult to say whether PPARδ is truly involved in promoting synthesis of the additional fat. Nevertheless, presence of additional hepatic triglyceride in GW501516 treated groups after 4 weeks was real and it could be also due to weakened β-oxidation processes in the liver. Still, no evidence was found to support this hypothesis. The pattern of hepatic expression of lipid oxidation marker gene suggested enhanced metabolism as shown by increases in the mRNA encoding CPT1 and UCP2. More global fatty acid catabolism upon feeding the animals with diet supplemented with GW501516 was also supported by examples of genes expression in skeletal muscle. Taking under consideration that PPARδ agonist action is not limited to liver, as the expression of PDK4 and UCP3 shows, and the fact that there is no solid proof for *de novo* lipogenesis or impaired β-oxidation in the liver in this study, it might be concluded that presence of additional fat in livers of GW501516 treated animals can come from peripheral sources like adipose tissue, which is highly likely due to the rapid weight loss in these animals. The possibility of shifting the distribution of fatty acids from adipose tissue to ectopic accumulation upon various stimuli was recently supported in work done by Zhong et al [145]. They showed evidence for contribution of alcohol-provoked adipose tissue lipolysis to hepatic steatosis. Rapid free fatty acids release from subcutaneous or visceral fat stores upon powerful stimulus might be actual cause or significant contribution to fatty liver phenotype.

These experiments were carried out to gain global picture on action of the GW501516 in non-transgenic mice and test hypothesis whether PPARδ agonist might promote fat accumulation in the mouse liver. However, to get

more insight into the actual cause of this phenomenon and determine if PPARδ receptor is solely responsible for the observed hepatic fat fluctuations in response to GW501516, we proceeded to utilise a transgenic animal models that would allow genetic alterations of PPARδ activity.

Chapter 4 Effects of dietary GW501516 administration in non-transgenic or human PPARδ or dominant negative derivative of human PPARδ mice on fatty liver phenotype.

4.1 Introduction

Rodent models for studying peroxisome proliferator activated receptors biology have been so far both useful and troublesome [146]. Carcinogenicity and hepatomegaly found in rodents treated with synthetic PPARα ligands is in contrast to primates and especially to men, since humans do not exhibit the liver toxicities associated with the rodent models treated with PPARα ligands.

Therefore, it is essential to find suitable animal models as close as possible in drug response to men, to foresee potential outcomes in humans. Since the primate model is not widely available and the rodents are prone to their species related differences in drug response, transgenic humanized mice or rats could turn out to be a promising alternative. In the study shown in the previous chapter we found that the PPARδ was the least abundant subtype of the whole PPAR family expressed in the liver of non-transgenic mice, with PPARα expression overwhelming the remaining 2 members of PPAR family (data not shown). Ideally, a humanized transgenic mouse model should reflect proportions of PPAR family found in the human liver, where there is no such disproportion in expression of PPARα over PPARδ [93]. Recently, 2 such models have appeared, including one used in the following study. Two approaches have been utilized when making the mouse transgenic model for human PPARδ. First in the work done by Gross et al [147] and second by our group [117,140]. In the first case, mouse endogenous PPARδ was simply replaced by human counterpart. The authors termed the model PPARδ knock-in

(PPARδ KI), which reflects genetic alternation done in the mouse. Summarizing the phenotype of their humanized mouse model for PPARδ, they show the ability of human PPARδ to replace the function of mouse PPARδ. Using the PPARδ other available specific activator, GW0742, they have shown that mouse and human PPARδ have overlapping functions in lipid and lipoprotein metabolism due to regulation of similar gene pool. However, the level of expression of the swapped receptor in livers of PPARδ KI animals was actually lower than endogenous PPARδ in non-transgenic mice. Although as they claim functionality of this nuclear receptor subtype between these 2 species was similar, it still doesn't reflect the proportions between PPARs found in human liver.

Our model of human PPARδ (hPPARδ) used in the following study (described in detail in methods chapter), not only allowed tracking of the physiological and transcriptional response of human gene in mouse model, but also mimicked more closely proportions of PPARs in human liver. Additionally, the dominant negative version of hPPARδ (hPPARδΔAF2) was used. This classical dominant negative deletion of the Activation Function 2, AF2, domain of PPARδ shows both constitutive transcriptional repression in addition to enhanced repression of PPRE signalling in the presence of a PPARδ agonist [148]. It serves as a loss of function or parallel-reversed model (when compared with the hPPARδ expressing mice) to help to decipher involvement of PPARδ receptor in hepatic steatosis observed in non-transgenic mice upon stimulation with GW501516 and described in previous chapter.

4.2 Results

Seventy 10-week old C57BL/6 background (30 non-transgenic mice; 10 mice expressing human PPARδ, and 30 mice expressing a dominant negative derivative of PPARδ (hPPARδΔAF2) – 30 mice) were placed on control (repelleted RMI + 0.25% I3C) or GW501516 (repelleted RMI + 0.25% I3C + 0.0025% GW) supplemented diet.

After two weeks, 30 mice (10 from each genetic group – 5 on control, 5 on GW501516 diet) were sacrificed. Then after 4 weeks from beginning of the experiment 20 mice (non-transgenic and hPPARδΔAF2) were taken. After 8 weeks of the experiment remaining 20 mice (non-transgenic and hPPARδΔAF2) were sacrificed.

All animals were sacrificed using increasing concentration of CO_2. Blood were removed using cardiac puncture, followed by organ removal (liver, muscle). Before the kill, animals were fasted to ensure adequate serum lipids measurements.

hPPARδ over-expressing animals were only included in the experiment for the first 2 weeks due the development psoriasis-like skin disease with more prolonged treatment with GW510516 [117,119].

After 14 days of treatment, weight measurements demonstrated that ligand treated hPPARδ animals lost 15% of the body weight in 2 weeks, when compared to untreated controls ($P<0.001$). Non-transgenic mice fed diet containing GW501516 and animals conditionally over-expressing hPPARδΔAF2 with GW501516 in diet, had no significant changes in body mass in relation to their control groups or maintained normal weight gain within 2 weeks time (Figure 4.1A). In the later stages of the experiment, involving this time only non-transgenic and hPPARδΔAF2 animals, wild type mice fed diet supplemented

with GW501516 had similar pattern of weight gain (Figure 4.1B) described in experiment from previous chapter, were animals were having no I3C in the diet. On the other hand, the animals over-expressing hPPARδΔAF2 were completely immune to GW501516-induced weight loss throughout the duration of experiment (Fig 4.1C). However, when comparing control mice only, the hPPARδΔAF2 animals had slightly lower weight gain than the non-transgenic mice in 8 weeks study (Fig 4.1 B and C), although the overall effect was not statistically significant.

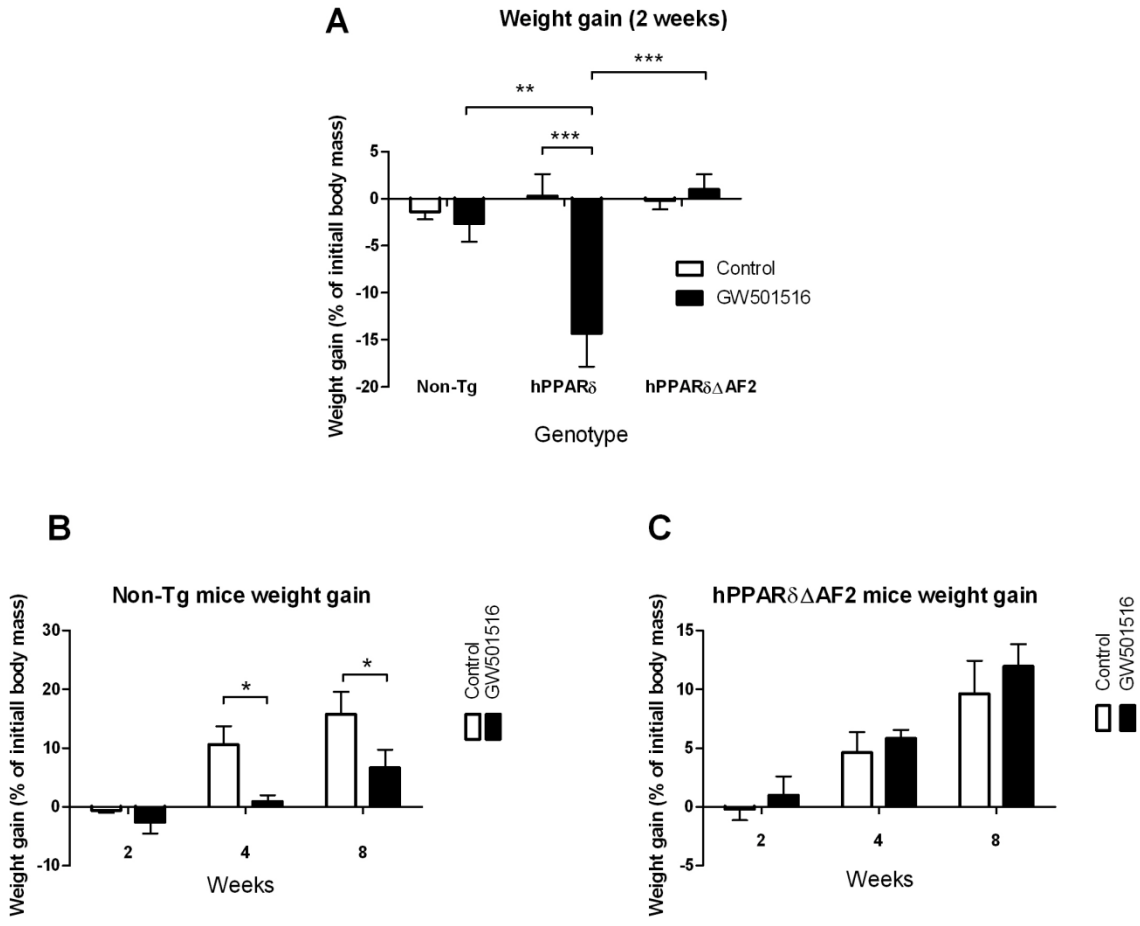

Figure 4.1 Body mass change in non-transgenic, hPPARδ and hPPARδΔAF2 mice. Comparison of body weight gain after 2 weeks, in all 3 genotypes (A). Differential weight gain in non-tg animals throughout the experiment (B). No mass change was

found between groups in hPPARδΔAF2 animal branch (C). Two-way ANOVA, significance is indicated as (*p<0.05; ** p<0.01; *** p<0.01), n=5 mice/group. Data are expressed as means ± SEM.

In similar a experiment with identical animal numbers and design, but ending after 12 days and done exclusively to obtain data from Medical Resonance Imaging (MRI), identical pattern of body mass change was found in all 3 genotypes as in 2-weeks time point (Fig 4.2A).

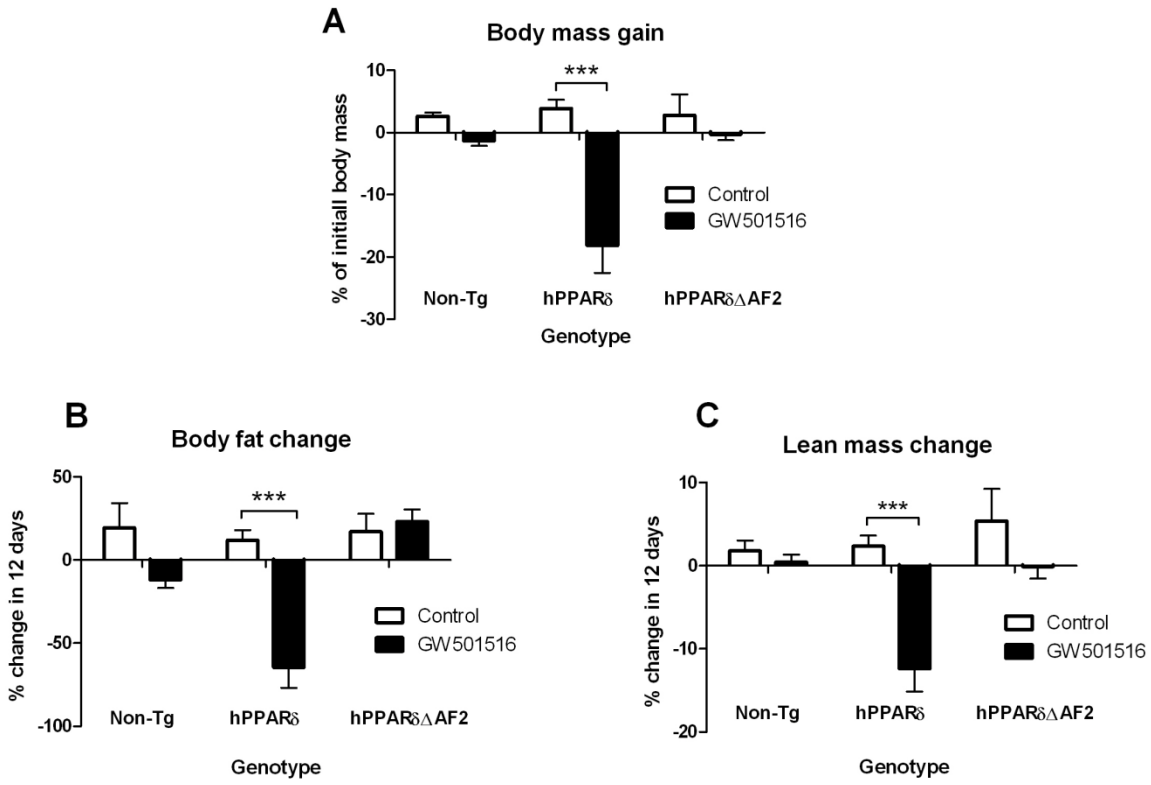

Figure 4.2 Results from whole body MRI scans of non-tg and transgenic animals fed control or diet supplemented in GW501516. Body mass change in 12 days of treatment (A). Percentage change in fat mass (B). Percentage change in lean mass (C). Two-way ANOVA, significance is indicated as *** p<0.001, n=5 mice/group. Data are expressed as means ± SEM.

MRI scans of the whole live animals revealed major changes in fat and lesser changes in lean mass compositions between first and last day of the experiment. Whereas all control groups increased body fat mass, non-transgenic animals fed diet supplemented GW501516 lost 12% of the body fat, hPPARδ mice on the same diet lost 64% of the body fat, while hPPARδΔAF2 animals fed diet with GW501516 gained 23% in fat (Fig 4.2B). There was virtually only one group, which had notable loss in lean mass, hPPARδ animals fed diet enriched in GW501516, which showed a 12% reduction of body lean mass. High association was found between body mass changes and fat or lean mass fluctuation. Although the correlation between lean mass and fat mass was lower, than seen for body mass vs lean mass (R^2=0.5566 vs R^2=0.9035), tests for correlation turned out to be extremely significant for both cases (P<0.0001) (Figure 4.3).

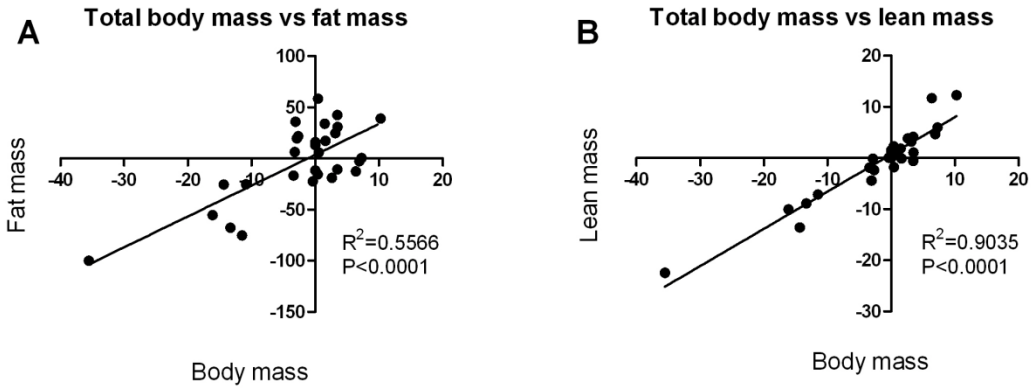

Figure 4.3 Correlations between total body mass vs fat mass (A) and between total body mass vs lean mass (B). Each point on the graph represents an individual animal used in this experiment (total number = 30) (12 days study).

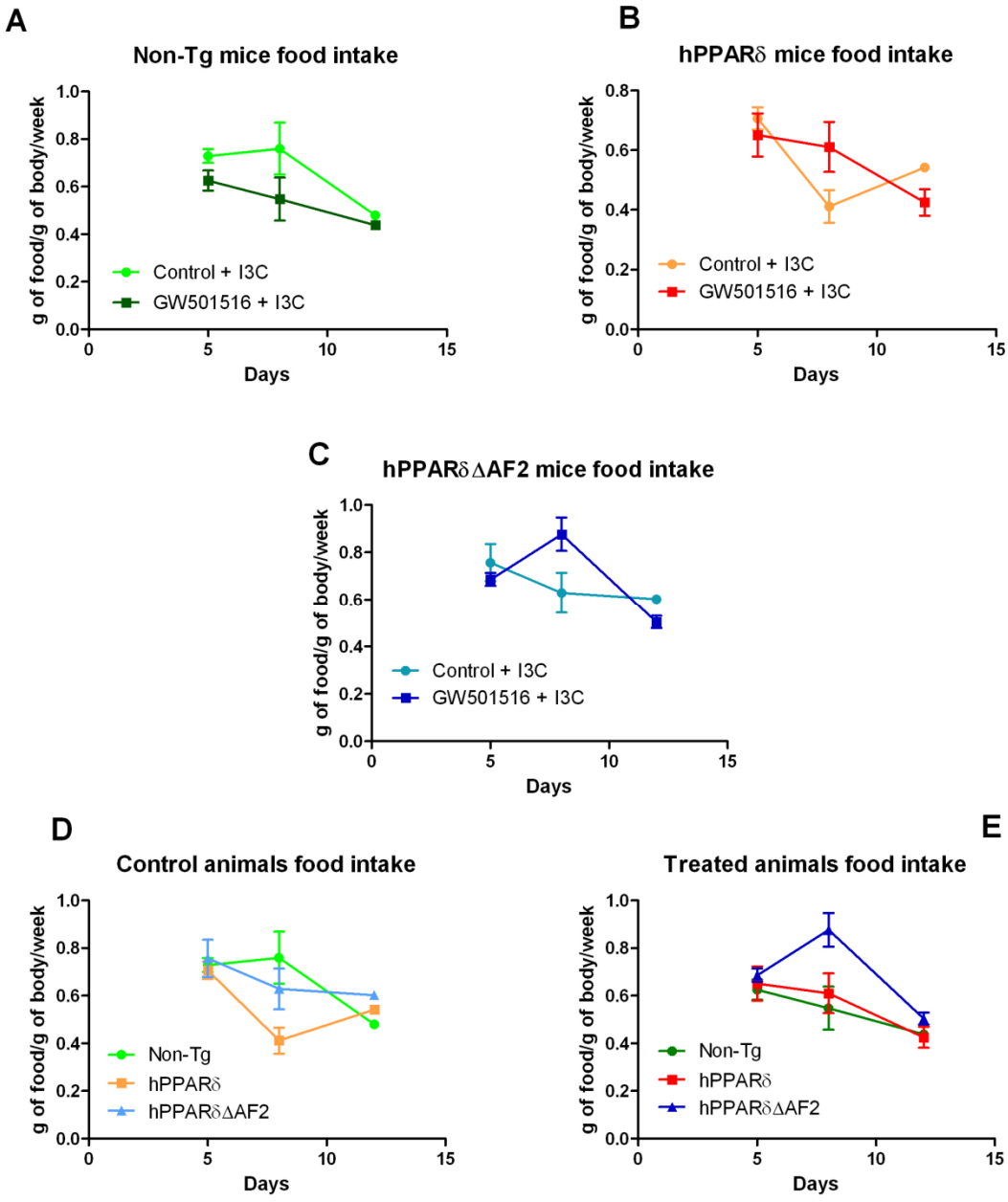

Figure 4.4. Food intake in 12 days experiment in non and transgenic mice. Consumption of food by non-transgenic mice (A); Food consumption by hPPARδ animals (B); Food intake by hPPARδΔAF2 mice (C). Comparison of food intake by controls of all 3 genotypes (D), and by treated mice (E), n=5 mice/group.

Food intake monitoring in the 12-days experiment showed that in non-transgenic animals, mice fed diet enriched in GW501516 only marginally consumed less than the control animals (Fig 4.4A). hPPARδ mice fed diet

supplemented with GW501516 initially consumed more than their controls without the ligand in the food, but at the end of the experiment situation has reversed (Fig 4.4B). The food intake levels in hPPARδΔAF2 animals was similar to that observed the hPPARδ animals (Fig 4.4C), despite striking differences in weight gain between these two genotypes upon GW501516 treatment.

Blood lipid measurements in 14-days experiment across all three genotypes have shown similar pattern as that seen in the study conducted in non-transgenic mice only. Total cholesterol was generally higher in non-transgenic and hPPARδ treated animals than in the controls, however, none of the differences turned out to significant (Figure 4.5A). No significant effect on plasma triglyceride was noticed in any of the genotypes although in hPPARδ there was tendency for lower triglyceride levels in treated mice (Fig 4.5B). Significant difference was found in HDL plasma levels derived from hPPARδ animals (Fig 4.5C). Treated mice had 52% higher levels of HDL than controls. No changes were observed in HDL levels in hPPARδΔAF2 animals. In non-transgenic mice treated with GW501516, HDL levels were still higher than in controls, but the difference was not statistically significant. Plasma free fatty acids (FFA) were higher in non-transgenic treated mice and control hPPARδΔAF2 animals when compared to their control or treated groups respectively. However, none of these changes were statistically meaningful (Fig 4.5D).

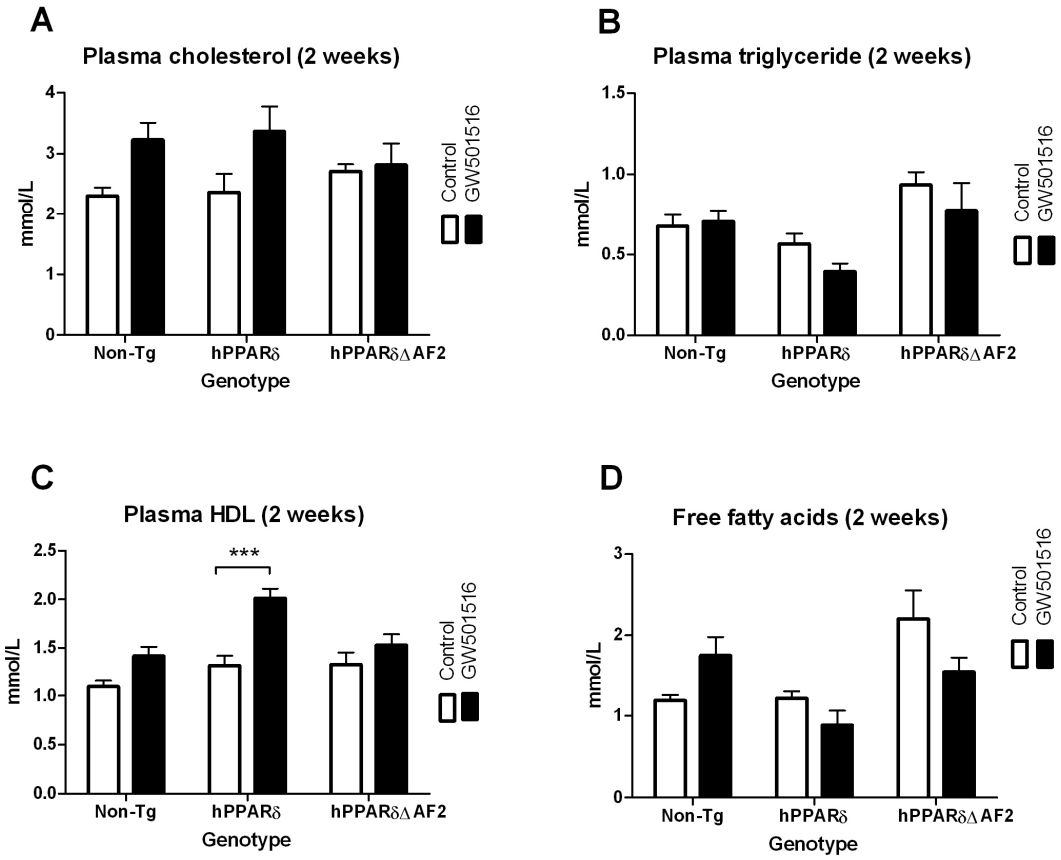

Figure 4.5. Plasma lipid profiles in non-transgenic and transgenic animals, after 14 days of GW501516 treatment. Plasma total cholesterol tendencies to rise upon GW501516 treatment in non-transgenic and hPPARδ animals were not found to be significant (A). No differences after 2 weeks was found in triglyceride levels (B). Extremely significant rise in HDL levels were detected in hPPARδ in mice fed diet enriched in GW501516 (C). Fluctuations in FFA were not translated into statistical significance despite observed tendencies (D). Two-way ANOVA, significance is indicated as *** $p<0.001$, n=5 mice/group. Data are expressed as means ± SEM.

Plasma leptin levels showed high variation among the genotypes. In non-transgenic PPARδ ligand treated animals lower levels of this adipose tissue-borne hormone were observed, although there was no statistical difference in this group. In hPPARδ mice fed diet supplemented with GW501516 leptin levels

were barely detectable when compared to controls fed normal diet and the difference turned out to be extremely significant (P<0.001). On the other hand the GW501516-treated hPPARδΔAF2 animals showed no decrease in blood leptin levels comparing to their controls (Fig 4.6A). Insulin (Fig 4.6B) and glucose (Fig.4C) levels showed no variation across genotypes or treatment groups.

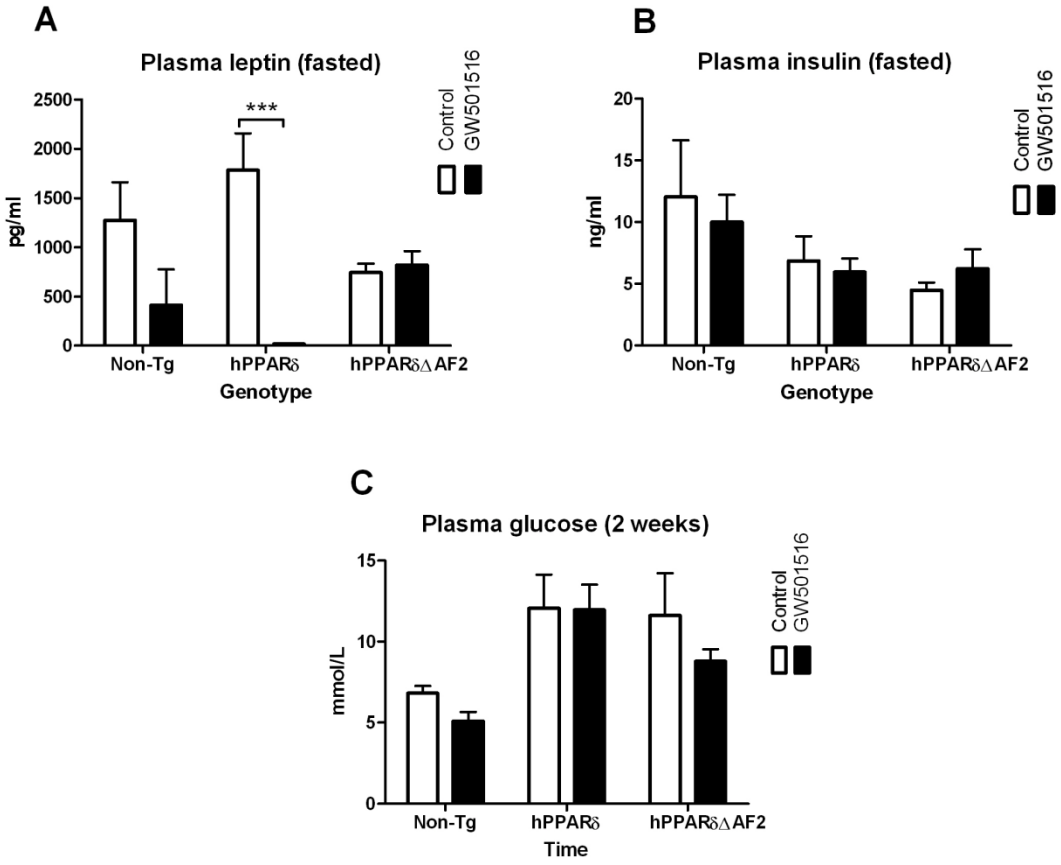

Figure 4.6.Blood hormones and glucose levels in 3 different genotypes treated with PPARδ ligand. Plasma leptin was almost eliminated in hPPARδ animals by GW501516 treatment (A). Insulin levels were not changed upon PPARδ agonist (B). No glucose variations between treatment groups were found (C), n=5 mice/group. Data are expressed as means ± SEM.

Following sacrificing the mice after two weeks, dissection of the animals revealed pale colour of the livers of hPPARδ and hPPARδΔAF2 mice.

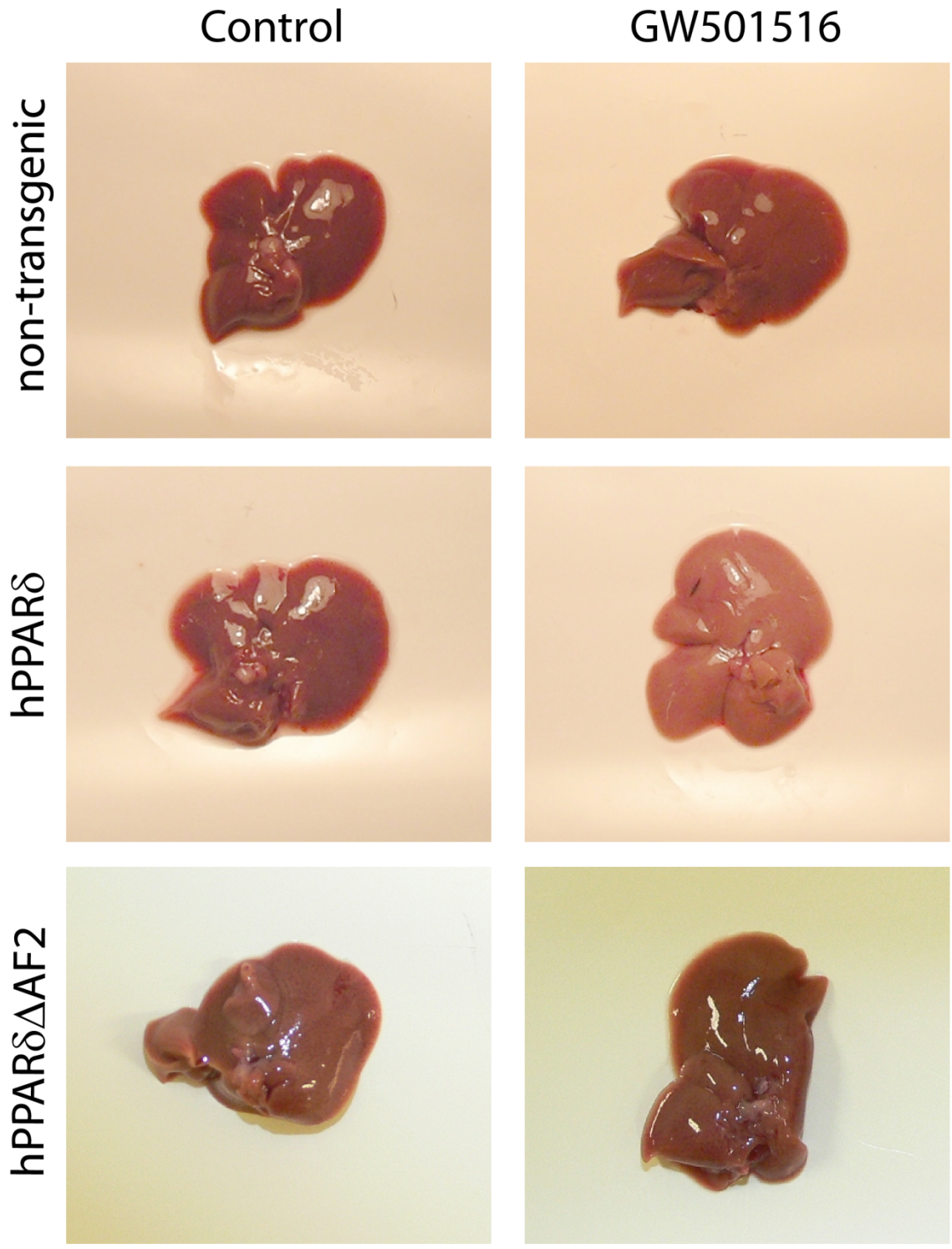

Figure 4.7 Examples of typical livers derived from mice fed for 14 days control diet or diet supplemented with GW501516.

Direct measurements of triglyceride in liver tissue showed that after 2 weeks non-transgenic mice fed diet enriched with GW501516 had slightly elevated hepatic triglyceride content when compared to their controls, although the difference turned out to be not significant. In mice over-expressing hPPARδ and fed diet supplemented with GW501516 liver fat content increased by 202% in comparison to their controls ($P<0.001$). Mice over-expressing a dominant negative form of hPPARδ (hPPARδΔAF2) with the ligand in the diet, accumulated 52% less fat in the liver, than control animals, ($P<0.001$). However, hPPARδΔAF2 control mice had hepatic fat content comparable to hPPARδ GW501516-treated group (Fig 4.8A). When comparing only treatment groups, hPPARδ mice had 109% more liver triglyceride than non-transgenic ($P<0.001$) and 85% more than hPPARδΔAF2 mice ($P<0.01$). (Fig4.8A). Cholesterol liver content after 14 days of experiment was only significantly elevated in hPPARδ animals fed diet containing GW501516 were difference between controls and treated animals reached 94% ($P<0.001$) (Figure 4.8B). Non-transgenic mice and hPPARδΔAF2 animals have revealed further fluctuations in hepatic fat content in later stages of the study, after 4 and 8 weeks. Wild type animal's hepatic lipid response to PPARδ ligand across all 3 time points resembled the one shown in previous chapter. There was a build-up of liver fat after 4 weeks in treated group, when compared to controls, with situation being reversed after 8 weeks of GW501516 treatment. In livers of hPPARδΔAF2 animals, triglyceride were at lower level in treated mice than in the animals fed control diet at all time point, however only at 2 and 8 weeks the difference was significant ($P<0.001$ and $P<0.01$ respectively) (Fig 4.8C).

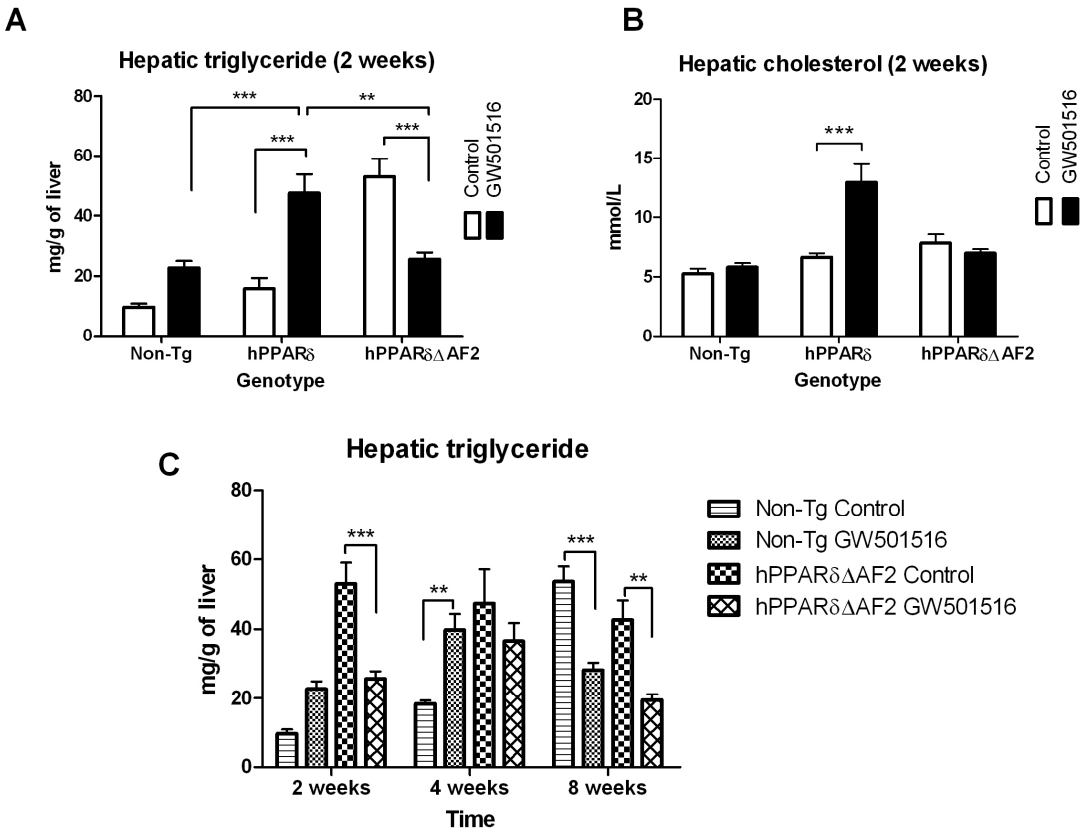

Figure 4.8 Hepatic lipid response in wild type and mice transgenic for hPPARδ and dominant negative hPPARδΔAF2 after feeding the control diet or diet supplemented with GW501516. Hepatic triglyceride after 14 days study in transgenic and non-transgenic mice (A). Liver total cholesterol was only significantly elevated in hPPARδ animals fed diet enriched in PPARδ agonist (B). Hepatic triglyceride across all 3 time points in non-transgenic and mice over-expressing hPPARδΔAF2. Two-way ANOVA, significance is indicated as ** $p<0.01$; *** $p<0.001$, n=5 mice/group. Data are expressed as means ± SEM.

RT-PCR quantification has shown that the expression of the transgene in transgenic animals in this particular experiment was significantly higher in the liver when compared to muscle expression (Figure 4.9), with no detection in tissues of non-transgenic animals. GW501516 availability did not affected

transgene expression pattern. Full data on transgene distribution across tissues and its inducibility was published before [140].

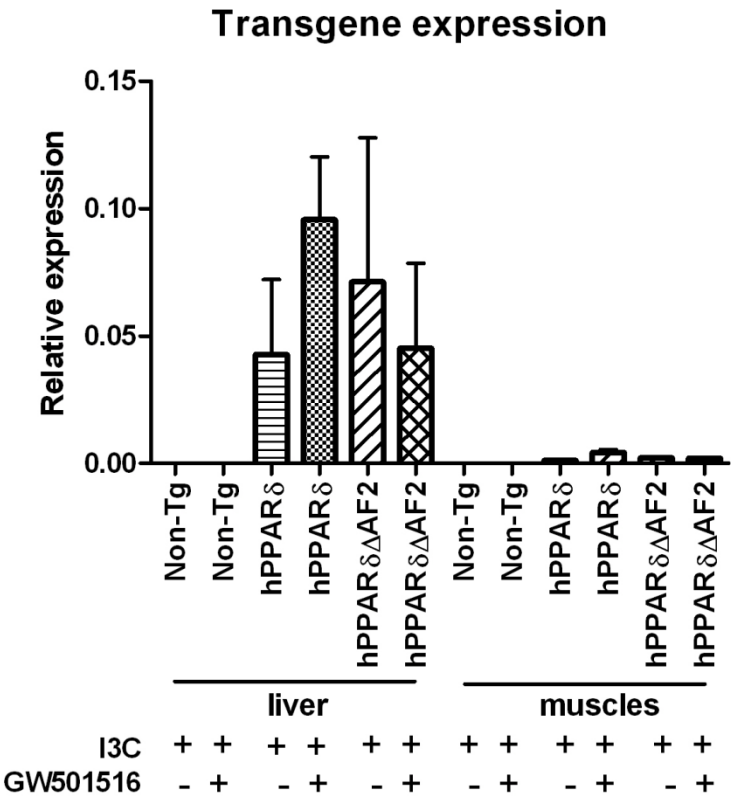

Figure 4.9 Human PPARδ and hPPARδΔAF2 induction in liver and muscle after 2 weeks feeding the diet supplemented with 0.25% Indole-3-carbinol. n=5 mice/group.

Patterns of hepatic expression of the members of PPAR family showed no significant differences between treatment groups, with exception of PPARγ being 3.5 fold up-regulated in hPPARδ control mice over treatment group ($P<0.01$). In muscle however, PPARγ showed no statistical variation in expression across the genotypes or treatment groups. This was in contrast to PPARα and PPARδ expression where there was significant difference between control and treatments groups in hPPARδ mice. PPARδ receptor was also up-regulated in hPPARδΔAF2 control mice, when compared to treatment group (Figure 4.10 A-F).

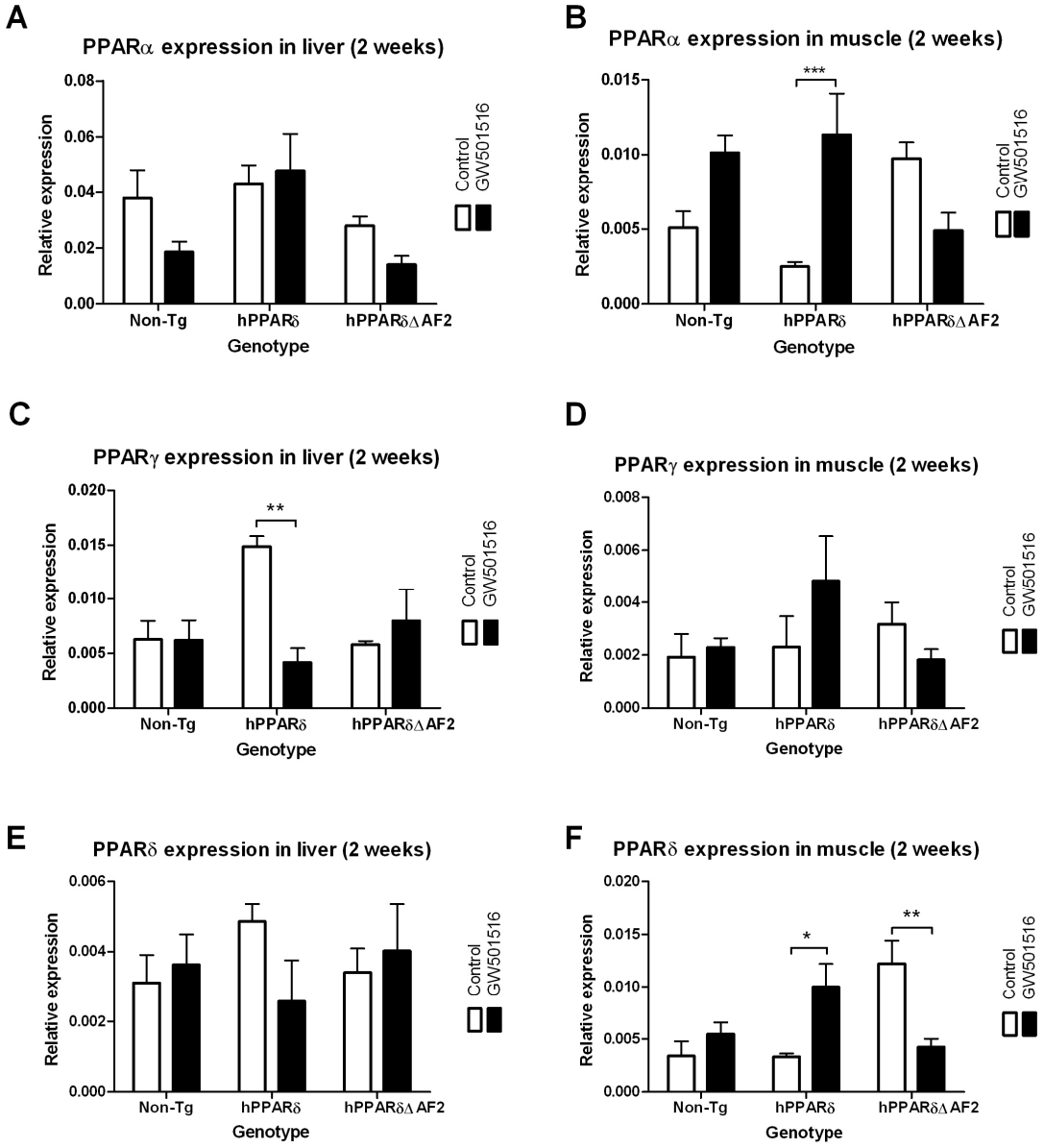

Figure 4.10 PPAR family expression in liver and muscle. PPARα in liver (A) and muscle (B), PPARγ in liver (C) and in muscle (D) and PPARδ expression pattern in liver (E) and muscle (F), n=5 mice/group. Data are expressed as means ± SEM.

Apolipoprotein C3 (Apoc3) levels, which are known to positively correlate with plasma triglyceride, across the genotypes after 2 weeks did not reach any significant difference between groups (Figure 4.11A). Similarly, HMG-CoA synthase, although showing trends involving PPARδ ligand and genotype in the

pattern of expression, none of these changes reached statistical significance (Figure 4.11B). Another PPARδ target gene PDK4, although primarily limited in tissue distribution to muscle, was also expressed in the liver where highly expressed in GW501516 treated group, however only in non-transgenic group. An almost identical pattern could be observed in muscle (Figure 4.11 C and D). Angiopoietin-related protein 4 (ANGPTL4) gene, which encodes a protein that functions as lipoprotein lipase inhibitor, has pronounced role in lipid metabolism, where it modulates triglyceride uptake and it is believed that it serves to protect a cell against cellular lipid overload by reducing extracellular TAG hydrolysis and consequent fatty acid uptake [149]. It is also known PPARδ target gene. However, it this experiment, in liver it was not differentially expressed between groups or genotypes and in muscle samples it was several fold induced by GW501516 in non-transgenic mice only (Figure 4.11 E and F).

Almost identical pattern of expression was observed in mRNA of ADRP in liver and muscle. The two Way ANOVA indicated a significant difference between the control and treated groups (Figure 4.12 A and B). Additionally expression of ADRP in the liver was highly associated with hepatic levels of triglyceride (Figure 4.13). Group means for liver TAGs and group means for ADRP showed high correlation regardless genotype or treatment status ($P=0.0073$ and $R^2=0.8636$). Another marker of lipid uptake, CD36 (Cluster of Differentiation 36) is a membrane associated protein and its mRNA was several fold over-expressed in livers of hPPARδ animals (Figure 4.12 C) and in muscle in non-transgenic and hPPARδ mice, however in the latter the difference was not considered significant (Figure 4.12D).

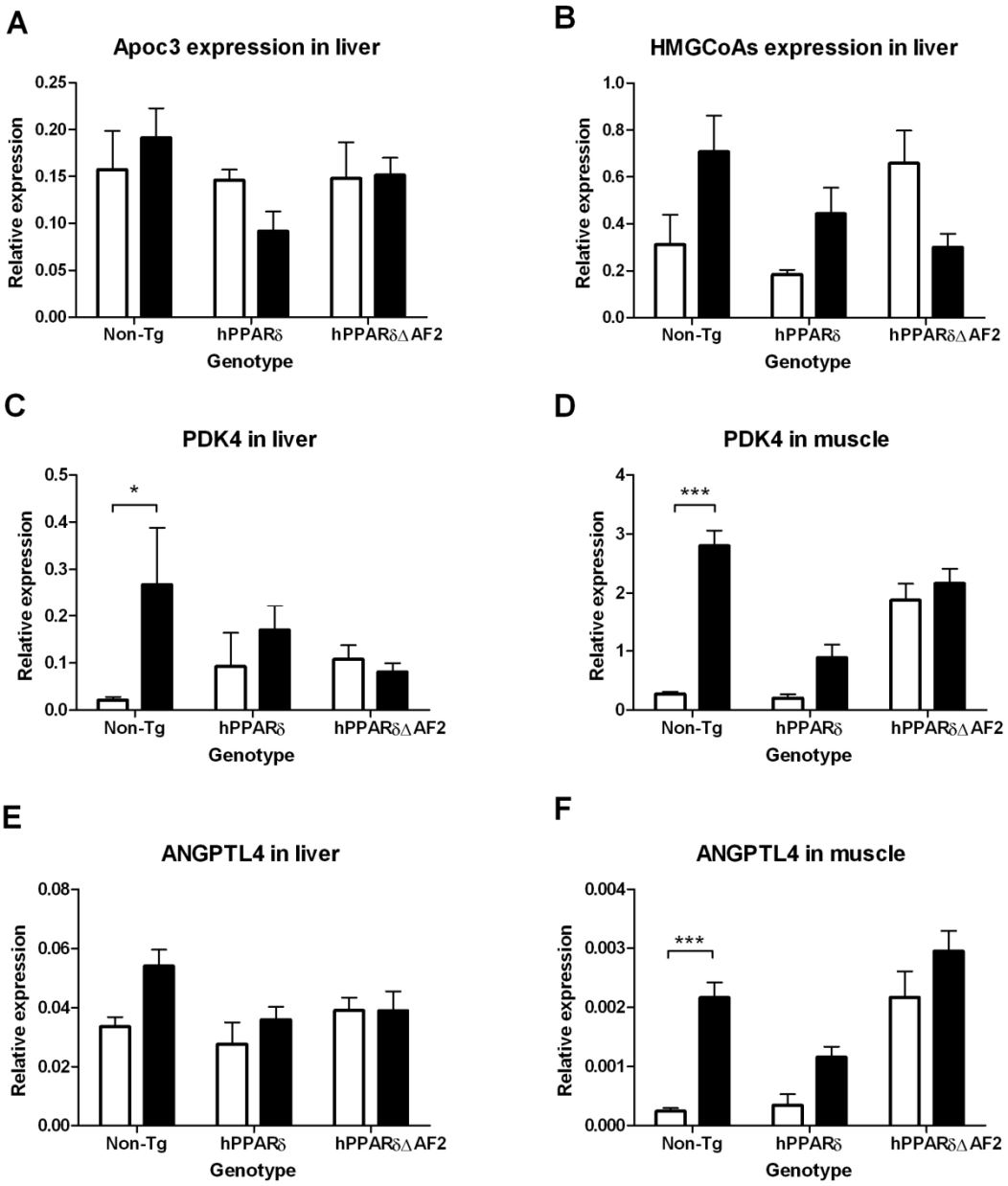

Figure 4.11 PPARδ target genes expression in liver and muscle. Apoc3 in liver (A), HMGCoA in liver (B), PDK4 in liver (C) and muscle (D) and ANGPTL4 in liver (E) and muscle (F). White bars – control groups, black bars - groups fed diet supplemented with GW501516. Two-way ANOVA, significance is indicated as * $p<0.05$; *** $p<0.001$, n=5 mice/group. Data are expressed as means ± SEM.

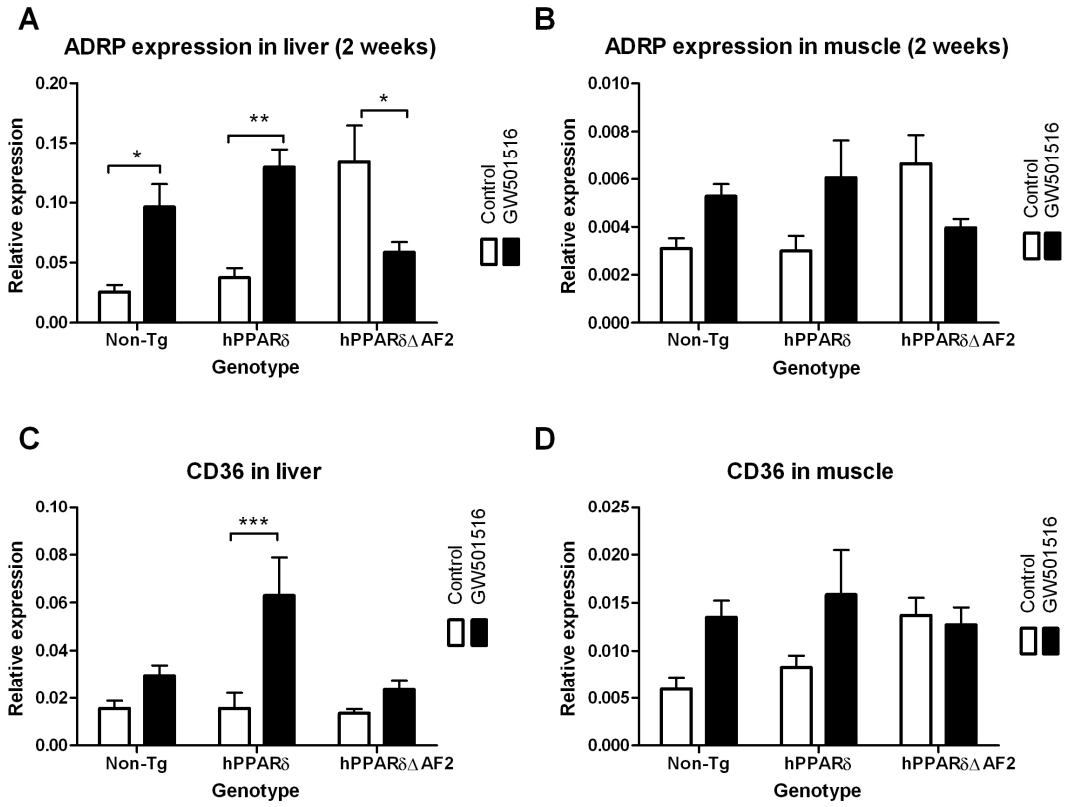

Figure 4.12 Expression of genes involved in lipid droplets coating and fatty acids uptake between the genotypes and treatment groups. ADRP in liver (A) and muscle (B) and CD36 in liver (C) and muscle (D). White bars – control groups, black bars - groups fed diet supplemented with GW501516. Two-way ANOVA, significance is indicated as * $p<0.05$; *** $p<0.001$, n=5 mice/group. Data are expressed as means ± SEM.

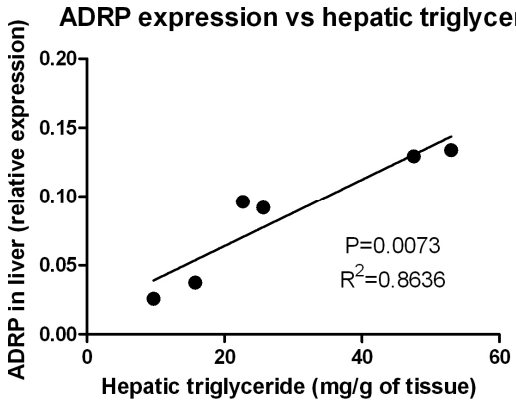

Figure 4.13 Expression of ADRP a protein marker of TAGs accumulation and direct PPARδ responsive gene, is significantly correlated with hepatic triglyceride levels.

Each point on the graph represents mean value of each group (control and treated) for ADRP relative expression vs mg/g of hepatic triglyceride.

Fatty acid synthase mRNA levels in liver showed no significant up-regulation in mice over-expressing human PPARδ; in fact, there was marked several fold down-regulation in non-transgenic and no effect of the GW501516 treatment on hPPARδΔAF2 mice. However in muscle, hPPARδ animals FAS mRNA levels indicated increased transcription of this gene in GW501516-treated animals when compared to their controls or to the wild type or hPPARδΔAF2 mice, regardless the groups status (Figure 4.14 A and B).

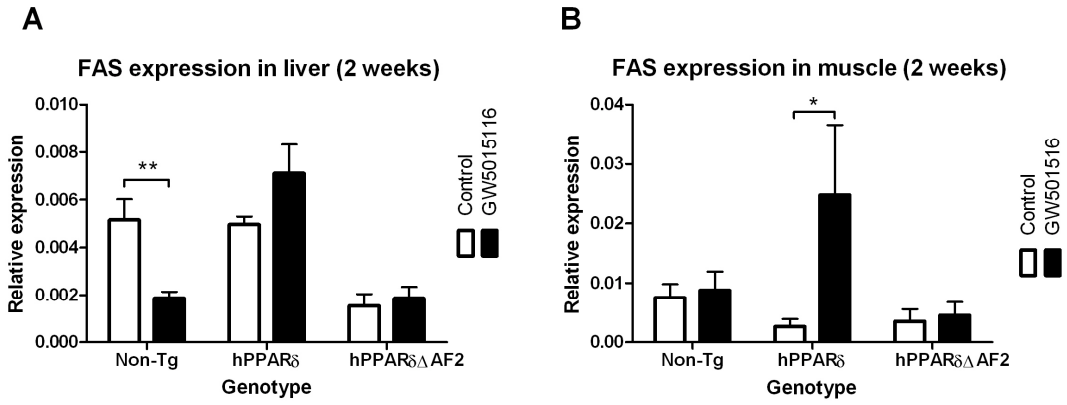

Figure 4.14. Fatty acid synthase expression across treatments groups and genotypes in liver (A) and in muscle (B). White bars – control groups, black bars - groups fed diet supplemented with GW501516. Two-way ANOVA, significance is indicated as *p<0.05; ** p<0.01, n=5 mice/group. Data are expressed as means ± SEM.

How the transgenes expression influences ß-oxidation process in this animal model is shown on the examples of 3 genes. Acox1 mRNA was increased by 214% in liver of GW501516-treated hPPARδ animals when compared to control group (P<0.01), and in muscle with trend to be GW501516-dependent

excluding hPPARδΔAF2 mice were the situation was reversed (Fig 4.15 A and B respectively). Additionally, hepatic expression levels of levels of CPT1 were not changed during the treatment in hPPARδ animals, but up-regulated in muscle upon agonist treatment (Fig4.15 C and D respectively). Uncoupling protein 2 (UCP2) is mitochondrial anion carrier and its function is to decrease reactive oxygen species production, it is also target of PPARs [150]. It can also serve as marker of energy turnover. In steatotic livers of hPPARδ mice it was markedly up-regulated (Fig 4.15 D) and in muscle also non-transgenic mice turned out to be responsive to PPARδ ligand. Only hPPARδΔAF2 animals were not affected by GW501516 in terms of of UCP2 mRNA levels.

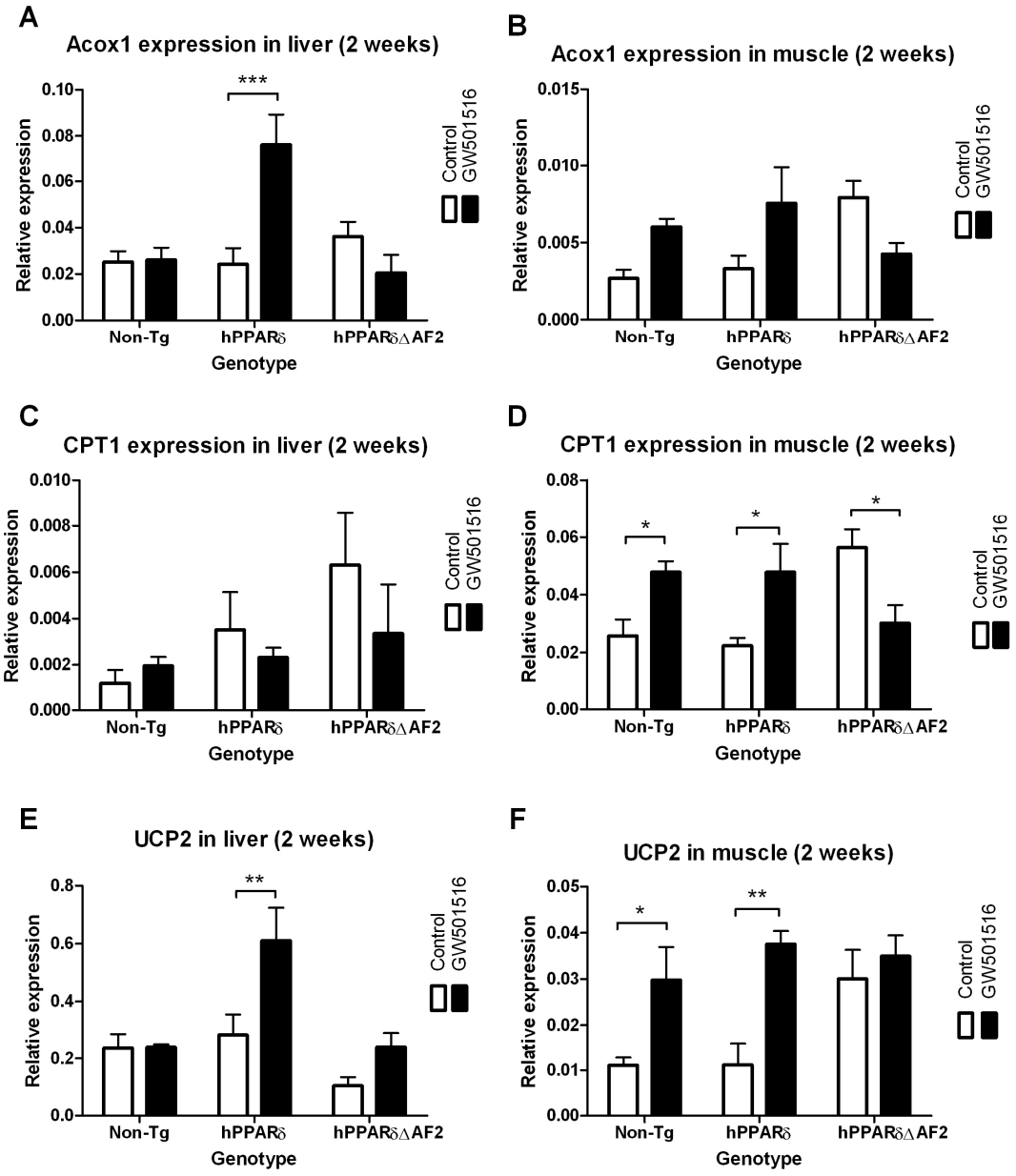

Figure 4.15 Expression markers of β-oxidation genes in non-transgenic, hPPARδ and hPPARδΔAF2 mice. Acox1 liver (A) and muscle (B) levels. CPT1 expression in liver (C) and muscle (D). And UCP2 hepatic E) and muscular (F) expression. White bars – control groups, black bars - groups fed diet supplemented with GW501516. Two-way ANOVA, significance is indicated as *p<0.05; ** p<0.01, n=5 mice/group. Data are expressed as means ± SEM.

4.3 Discussion

We wished to examine the role of PPARδ in the action of GW501516 that we observed in our studies in Chapter 1. In order to this we used both gain and loss of function strategies in transgenic mice. For a gain of function approach we used transgenic mice conditionally over-expressing human PPARδ and for a loss of function approach we used transgenic mice conditionally over-expressing dominant negative derivative of human PPARδ.

PPARδ receptor is responsible for GW501516-induced weight loss

Confirming our hypothesis, weight loss was particularly evident when hPPARδ animals were fed a diet containing GW501516 (Fig 4.1 A). And the dominant negative hPPARδ over-expression completely prevented weight reduction by GW501516. This convincingly confirmed the leading role of the PPARδ receptor in GW501516-related effects. Although fat disappearance was the main contributor to total body weight loss in non-transgenic and hPPARδ mice, lean mass was reduced too (Fig. 4.2 C). It might suggest role of the transgene activity in this process, however, hPPARδ was not induced in muscle of these animals [140] (Fig. 4.9). The evidence from semi-starvation experiments in humans also show that muscle mass loss is initiated in parallel to fat mass reduction from the very beginning of the reduction of caloric intake, although the latter is lost at much faster rate [33]. The correlation was stronger between total mass reduction and lean mass reduction rather that between total body mass versus fat mass in both transgenic models and the control animals. This imbalance comes from the fact that, hPPARδΔAF2 animals fed diet enriched

with GW501516 failed to mobilize their fat deposits, but in 12 days, they were still retarding the lean mass gain in the presence of PPARδ agonist (Fig 4.2 C).

Activity of PPARδ plays an important role in energy homeostasis regulation

Interestingly, while control non-transgenic mice in the current study had the trend to reduce the food intake when GW501516 was available in the diet, 2 weeks of studying physiological response to PPARδ agonist in transgenic mice showed no significant differences in food consumption, when compared to their genetic controls. This is also in contrast to what was described in previous chapter, where wild type mice had diminished appetite when GW501516 was added into the diet. It is not surprising considering the hPPARδΔAF2 mice, which represent the dominant negative model of the PPARδ receptor. However, regardless of the mechanism of appetite suppression by PPARδ agonist in wild type mice, it is not strongly pronounced in the hPPARδ animals. It is possible that basal reduction in food consumption is compensated by extensive nutrient-demanding metabolic state and therefore enforced increase in food intake, created by over-expression and activation of hPPARδ. PPARδ is the most abundant isoform in the central nervous system and is enriched in the hypothalamus, a region of the brain involved in energy homeostasis regulation. In study performed by Kocalis et al, neuronal deletion of PPARδ resulted in a susceptibility to diet induced obesity in animal model [151]. Their findings and our results on the involvement of GW501516 on appetite suppression, indicate that PPARδ plays an important role in energy homeostasis regulation which is independent of PPARδ-induced WAT lipolysis. Adipose born hormones like leptin, may play a significant role in this mechanism. There was virtually no

leptin in the plasma derived from hPPARδ animals fed diet enriched with GW501516 (Fig 4.6 A) and the leptin is known to play great role in modulating the appetite, with low blood levels stimulating the food intake [62]. Therefore we would expect the food intake of the GW501516 treated hPPARδ mice to increase. Extremely low levels of leptin in blood of hPPARδ mice, could be explained directly by significant reduction in adiposity preceded by over-expression of the transgene and activation by PPARδ ligand. Leptin was not affected by GW501516 treatment in hPPARδΔAF2 mice, which confirms potential of this receptor to modulate leptin levels [122]. Insulin levels in transgenic mice were consistent in not being influenced by PPARδ activity, similarly to what was found in wild type animals in previous chapter.

Lipid metabolism is regulated by PPARδ

Surprisingly, there was no decrease in plasma triglyceride in hPPARδ, nor in the wild-type mice in 2 weeks time. In previous experiment, this GW501516-associated effect was observed after 14 day of treatment in non-transgenic animals (Fig 3.3B). The interference of I3C signalling might not be excluded. Although the recent report shows that I3C itself possesses triglyceride lowering capability [152]. On the other hand, the effect on the HDL levels seemed to be pronounced in animals conditionally expressing hPPARδ, where only 2 weeks were enough to reach the levels of HDL, which in wild type animals, were only achieved after 4 weeks (Fig 3.3 D and Fig 4.5 C respectively).

In non-transgenic mice 2-weeks feeding with diet enriched in PPARδ agonist provoked only minor fluctuations in liver triglyceride (Fig. 3.5 and Fig. 4.8A), while in mice transgenic for hPPARδ, this time was sufficient for

accumulation significant amount of lipids in the liver. Higher level of hepatic triglyceride was also found in hPPARδΔAF2 animals on normal chow diet, but not in hPPARδΔAF2 mice with GW501516 in the diet. It might be rather surprising, as PPARδ in this case was not functional, because of lack of ligand-activated AF2 domain. However, recent findings [148,153] suggest that PPARδ main role could be a repressor function. Gustafsson et al concluded that genetic ablation of PPARδ will remove the intrinsic role that PPARδ has in the tempering of signalling of PPARα and PPARγ. Similar conclusion was drawn by already cited Adhikary et al study. On the other hand, findings described in this chapter show that hepatic fat level was reduced in hPPARδΔAF2 mice with the PPARδ agonist in the diet, being comparable to those found in non-transgenics after 2 weeks treatment. Exactly the same pattern could be seen in mRNA expression of ADRP in liver in 2 weeks (strong correlation with liver triglyceride, Fig 4.13) and also in other genes expressed in liver and in muscle (Figure 4.12 and 4.14). And animals over-expressing hPPARδΔAF2, which were fed for 8 weeks diet supplemented with GW501516 had minor fluctuations in hepatic fat, but the levels of fatty acids were always lower in this group, than in the control animals within the same phenotype. The non-transgenic animals in this experiment have shown consistency in the hepatic fat levels with the findings from previous chapter.

Concluding, ligand binding to AF2 domain-deficient PPARδ, restores repressing function of this nuclear receptor. In this case, agonist bound to AF2-incomplete hPPARδ, works in antagonist fashion, efficiently competing with endogenous mouse PPARδ for PPRE binding sites. This model of PPARδ

expression provides an additional tool, confirming association between PPARδ signalling and hepatic fat accumulation.

Liver is the key organ for PPARδ-associated effects

Endogenous PPARδ is expressed ubiquitously in most of the body organs [137]. Therefore, it is difficult to determine, which of them could be crucial for PPARδ-agonist linked regulation of plasma lipids, weight loss and liver steatosis. In our animal model however, the basal hPPARδ transgene expression was very low in range of tissues and highly inducible in the liver (Figure 4.9), [140]. Consequently, the pronounced weight loss and hepatic lipid accumulation in mice conditionally-expressing hPPARδ fed diet supplemented with GW501516, might lead to the conclusion that liver is vital for generation of this phenotype.

Despite the well established induction of the transgene in the liver, in this organ, PPAR family members did not show noticeable and significant relation to the genotype or treatment status. Unlike the liver, muscle PPARs, especially PPARα and PPARδ demonstrate pattern of expression, which is also evident in other genes. Namely, pronounced expression in wild type, hPPARδ treated with PPARδ agonist and hPPARδΔAF2 control animals. Examples of such pattern of expression can be found in ADRP and HMGCoAs in liver or CPT1, UCP2 and Acox1 in muscle. This set of the genes reflects more closely the repressor function of PPARδ, where ligand binding to endogenous mice receptor or hPPARδ or negative dominance of hPPARδΔAF2 relieves the repression, which drives the transcription of the target genes. Not all genes shown in this chapter however, manifest the repressor function of the PPARδ, which is consistent with

findings of Adhikary et al, where 3 differing modes of target gene regulation by PPARδ have been described. In these studies PPARδ was shown to control gene expression by; (a) type 1 response: up-regulation of the target genes by siRNA knock down of PPARδ, but not by GW501516, (b) type II response: up-regulation by knock down of PPARδ and by GW501516 binding, (c) type III response: down-regulation by PPARδ siRNA, but not by GW501516 binding [153].

As previously have been shown in wild type animals model, in hPPARδ mice the hepatic gene expression of fatty acid synthase (FAS) (Fig 4.14 A) or genes involved in β-oxidation (Fig 4.15) do not support the hypothesis that the origin of the additional fat in the livers in animals treated with PPARδ ligand comes from fatty acid *de novo* synthesis or crippled fatty acid burning and turnover. Some new light is shed on causes of observed massive hepatic lipid accumulation in animals treated with GW501516 by parallel substantial reduction of adipose tissue deposits and CD36 expression in liver. CD36, which supports transport of fatty acids across the cellular membrane, was markedly up-regulated in hPPARδ livers fed diet enriched with GW501516. The presence of additional hepatic fat in control hPPARδΔAF2 animals is however neither consistent with weight loss or CD36 up-regulation in these animals. Possibly, hPPARδΔAF2 transgene induction in this model is enough to release amount of fatty acids from adipose cells to cause hepatic steatosis, but insufficient to deplete the body of its adipose tissue, as it was found in mice over-expressing full length human PPARδ receptor and activated by the agonist.

All of the experimental mice used in this chapter, were healthy 10 weeks old animals fed normal diet. Since the PPARδ agonists are hoped to be used to

correct some aspects of the metabolic syndrome, it is important to gain a better understanding of hPPARδ activity in different metabolic contexts such as high fat diet and obesity. The next chapter aims to address this.

Chapter 5 Effects of chronic high fat dietary intake in mice conditionally expressing human PPARδ or dominant negative derivative of human PPARδ on body weight and hepatic triglyceride accumulation.

5.1 Introduction

One of the contributors to non-alcoholic fatty liver disease (NAFLD) is diet [36]. In general, saturated fat and fructose contribute to hepatic lipid accumulation, whereas unsaturated fat, choline, antioxidants and high-protein diets rich in isoflavones seem to have a more preventive effect [37,129]. Unsaturated fatty acids are also one of the activators of PPARδ [154]. Recommend consumption of fat by clinicians is defined as a diet deriving less than 30% of the energy from fat [33]. The typical laboratory experimental high fat diets (HFD) for rodents, have higher than 30% energy from fat, but usually contain balanced fatty acid composition. The one used in this study derives 60% of the daily calories from fat and has a ratio of total saturated fatty acids to monounsaturated fatty acids approximately equal to 1. In normal wild-type animals, long term feeding with HFD eventually leads to relative increase of adipose tissue mass, total body weight gain and possibly, liver fat accumulation [155,156]. Bearing in mind that monounsaturated fatty acids or their derivatives can activate PPARδ, serving as its partial agonists and on the other hand high content of saturated fat in HFD, feeding the mice over-expressing hPPARδ using this diet can have two possible effects. One would be the usual over-nutrition resulting in body mass gain and possibly liver steatosis, and alternative, protective role against diet induced obesity. Using our transgenic

model, we tested the role of hPPARδ in long term HFD feeding in obesity and liver steatosis.

5.2 Results

9-13 week old C57BL/6 mice (6 per group -3 males, 3 females) were placed on high fat diet (60% energy from fat) or on high fat diet + transgene inducer - I3C (0.25%(w/w)). Wild type control mice and two strains of transgenic mice were used: transgenic for human PPARδ receptor (hPPARδ) and dominant negative PPARδ receptor with blocked activation domain (hPPARδΔ2AF). GW501516 was not used in this study. After 18 weeks all mice were sacrificed. During the experiment, hPPARδ mice in the treated group did not develop symptoms of psoriasis-like skin disease that was seen in hPPARδ animals treated with GW501516. Throughout the experiment, wild-type mice and wild type mice fed diet supplemented with I3C had comparable weight gain rate with the I3C group in last 3 weeks putting on less weight (Fig 5.1 A). Nevertheless at the end of the experiment the difference in weight gain between the I3C and non I3C groups in wild type mice was not significant (Fig 5.2). In contrast, hPPARδ mice fed HFD with I3C after 4 weeks of study have stopped gaining weight reaching the plateau, which lasted almost to the last week of experiment (Fig 5.1 B). After 18 weeks on HFD, control group had 4.5 fold increase in percentage of weight gain over the normal chow group (P=0.0015) (Fig 5.2). The third branch of the experiment, hPPARδΔ2AF mice when fed HFD without I3C maintained steady weight gain throughout the time of experiment until the last week, but the HFD+I3C group had significantly lower weight gain rate starting from the second week of experiment (Fig 5.1 C).

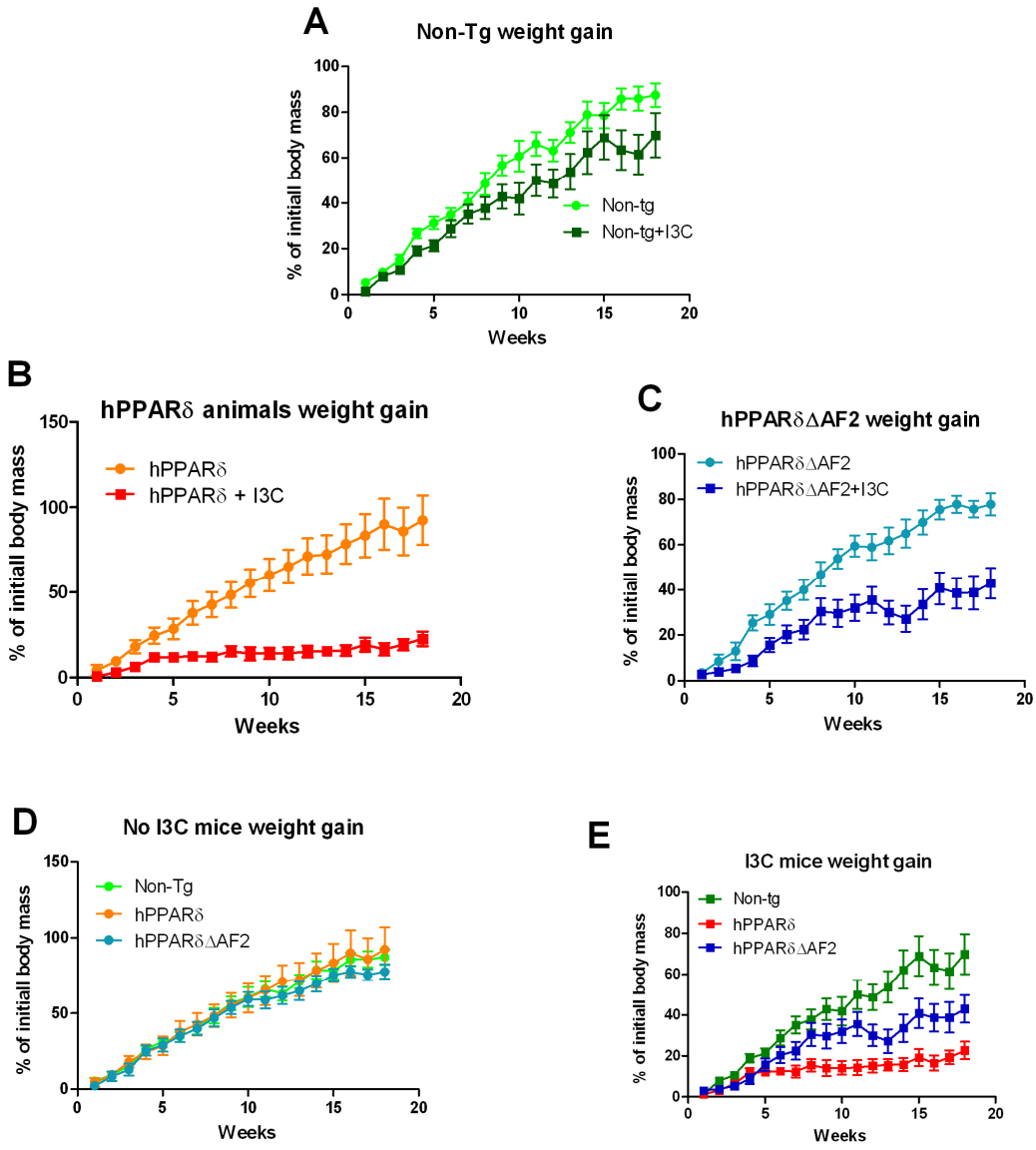

Figure 5.1 Weight gain of non-transgenic mice (A), hPPARδ mice (B) and hPPARδΔAF2 mice (C) fed plain HFD (D) or HFD supplemented with I3C serving as a transgene inducer (E). n=6 mice/group

At the end of the study, hPPARδΔ2AF animals fed HFD+I3C had significantly lower weight gain when compared to their genotypic controls (Fig 5.2), and also lower than non-tg animals fed HFD+I3C, but statistically higher than hPPARδ mice fed HFD+I3C (Fig 5.1E).

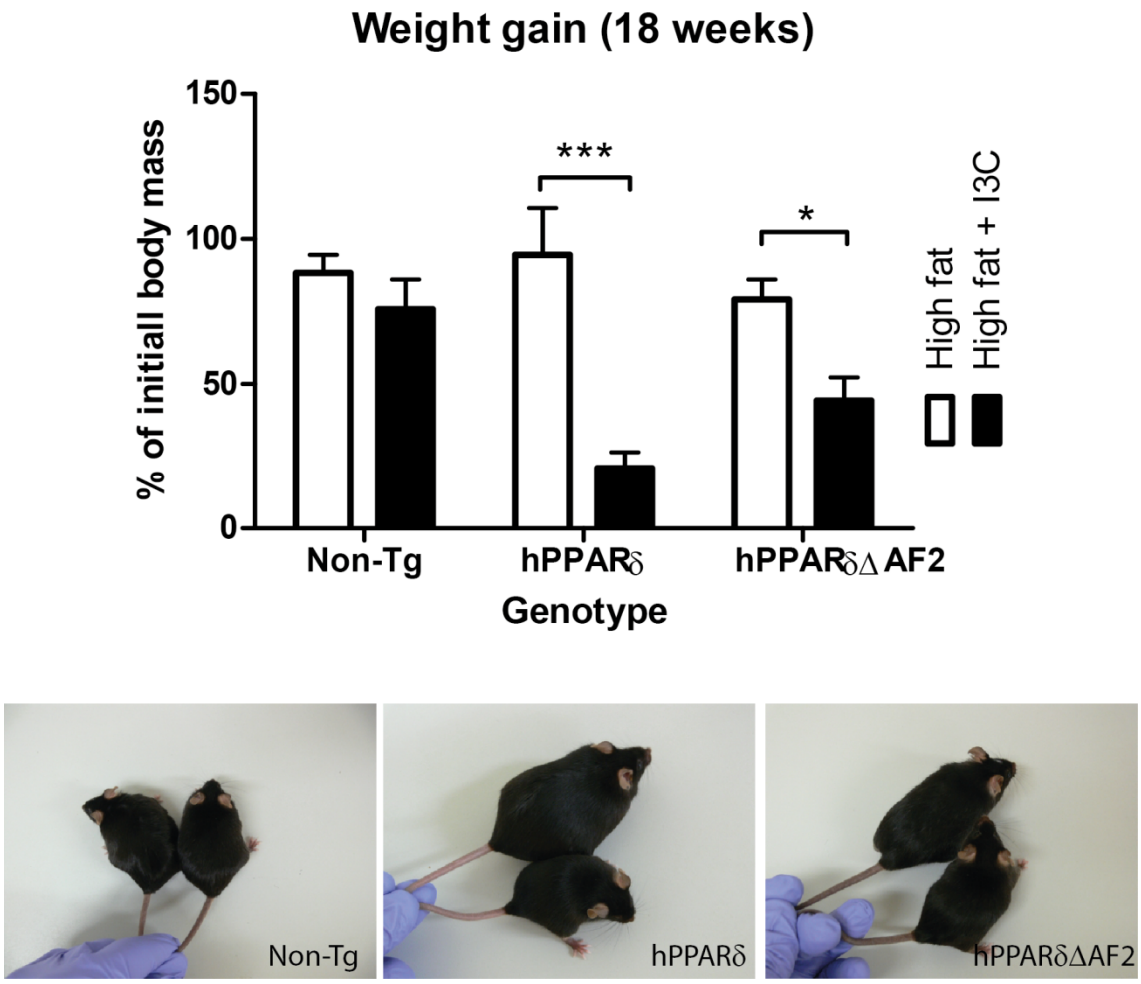

Figure 5.2 Weight gain data from last day of experiment of non-transgenic, hPPARδ and hPPARδΔAF2 mice fed plain HFD (D) or HFD+I3C. Pictures show the proportion of the body size in relevant mice in all three genotypic groups. Mice presented on the pictures from left hand side are examples of control animals, right hand side – mice from I3C groups. n=6 mice/group. Data are expressed as means ± SEM.

The weekly food consumption was also measured throughout the duration of the experiment. Surprisingly, the food intake could not be treated as an indicator or explanation of weight gain, as in the case of non-transgenic and hPPARδ mice, animals with the I3C in the diet periodically consumed more than those on plain HFD. hPPARδΔ2AF animals dietary groups had no difference in food

intake, although like the rest of the animals there was clearly noticeable trend in decreasing food consumption over time (Fig 5.3).

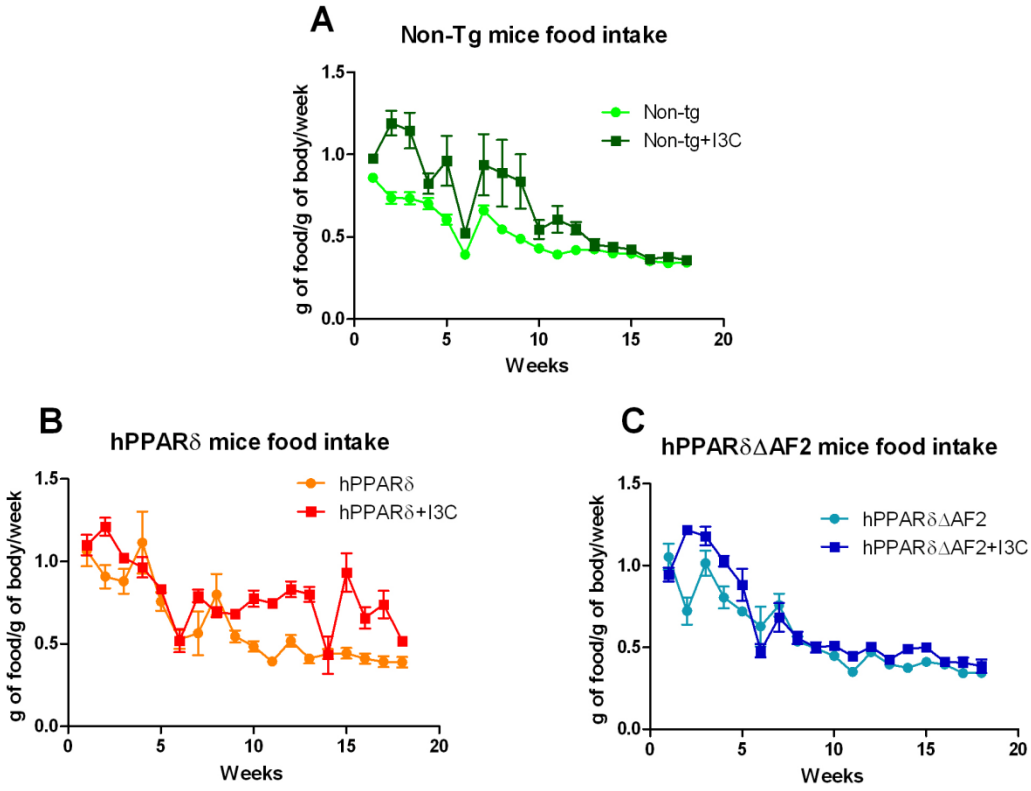

Figure 5.3. Food consumption in high fat diet experiment among non-transgenic (A), hPPARδ (B) and hPPARδΔ2AF mice. n=6 mice/group

After dissection of the hPPARδ animals, enlarged, visceral fat pads were exposed in animals fed HFD only. In hPPARδ fed HFD supplemented with I3C, visceral fat was still visible, although not well-defined as those seen in control animals (Fig 5.4). The visceral adiposity of wild type animals and hPPARδΔ2AF was comparable to the one found in hPPARδ mice fed plain HFD (data not shown).

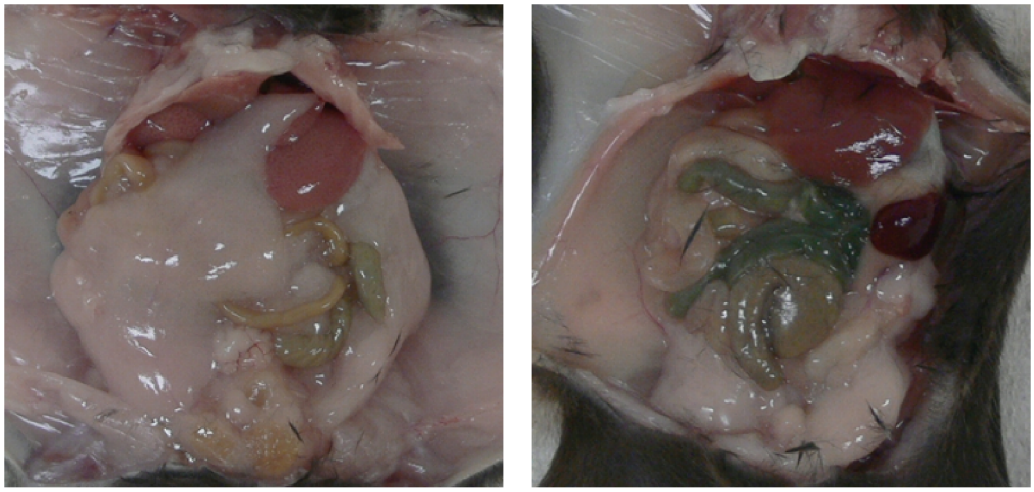

Figure 5.4 Visceral fat pads exposed post-mortem in hPPARδ mice fed plain HFD (left hand side picture) and fed HFD I3C (right hand side picture).

Plasma lipids including triglyceride, total cholesterol and HDL did not show any noticeable differences between dietary groups or between genotypes (Figure 5.5), although when corrected for gender, hPPARδ males in HFD+I3C group had significantly higher HDL levels than the control group fed plain HFD (data not shown).

After 18 weeks, hepatic triglyceride levels in non-tg animals groups were comparable. In mice conditionally-expressing hPPARδ, hepatic triglyceride in HFD+I3C group were lower by 68%, when compared to control group fed plain HFD (P=0.001). Animals over-expressing the dominant negative hPPARδ, also had a lower amount of liver fat than their controls, but the difference was not considered statistically significant (Fig. 5.6A). Interestingly, hepatic cholesterol was on equal level in all but one group. hPPARδ mice fed HFD+I3C, had elevated levels of intrahepatic cholesterol, but this phenomenon was gender specific with only 2 females (per 3 females per 6 animals in the group). Therefore statistical test did not considered this finding significant (Fig 5.6B).

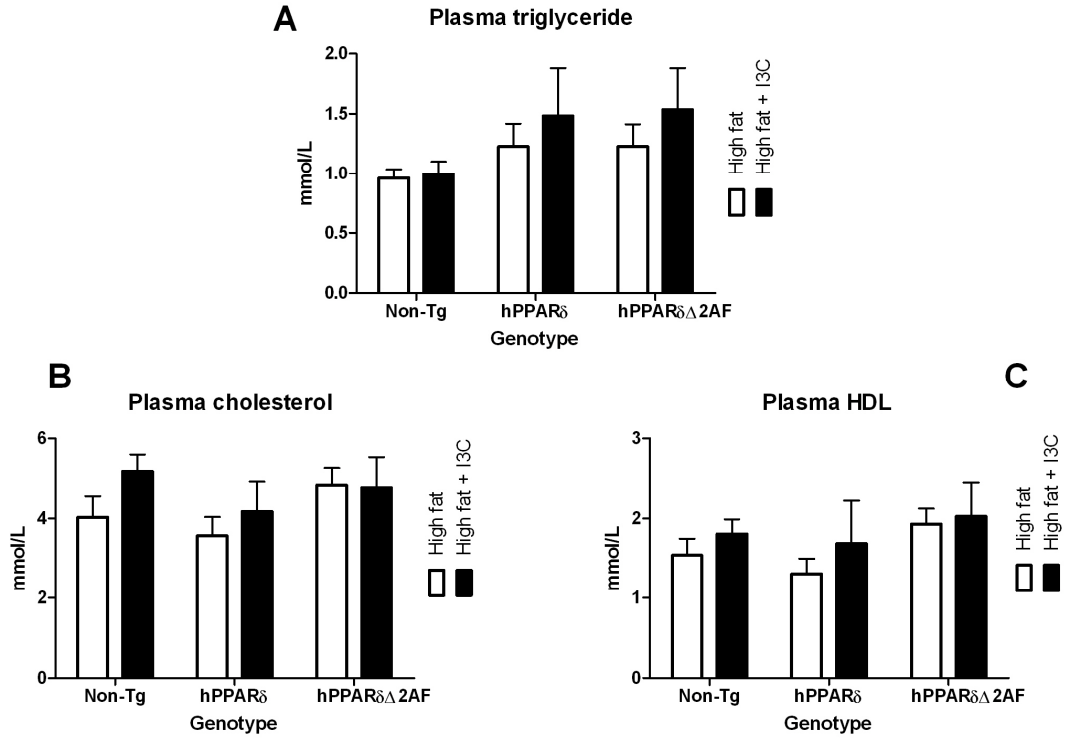

Figure 5.5 Plasma lipids in mice fed HFD or HFD+I3C. n=6 mice/group. Data are expressed as means ± SEM.

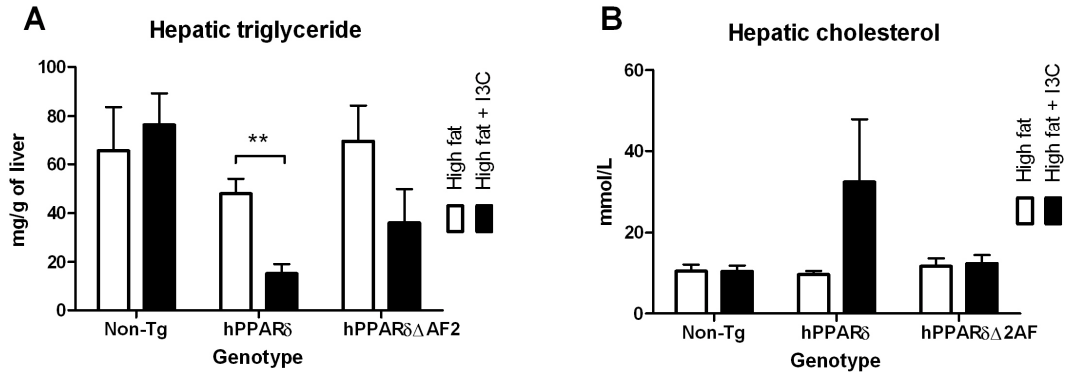

Figure 5.6 Hepatic triglyceride (A) and hepatic total cholesterol (B) in wild type and transgenic animals fed HFD or HFD+I3C for 18 weeks. n=6 mice/group. Data are expressed as means ± SEM.

To test whether the observed important inter-genetic changes in rate of weight gain and hepatic lipid accumulation were indeed caused by induction,

and subsequent activity of the transgenes, key metabolically active tissue were screened using RT-PCR for expression of the hPPARδ and hPPARδΔAF2. The basal levels of the transgenes were very low in every screened organ. White adipose tissue (WAT), brown adipose tissue (BAT) and muscle did not show signs of any induction of transgenes. In liver, contrary to the three first organs, although basal levels of the hPPARδ and hPPARδΔAF2 were low, with magnitude comparable to previous 3 organs, in presence of I3C, the transgene expression was induced 321 and 434 fold (hPPARδ and hPPARδΔAF2 respectively), when compared to animals fed plain HFD (Fig 5.7).

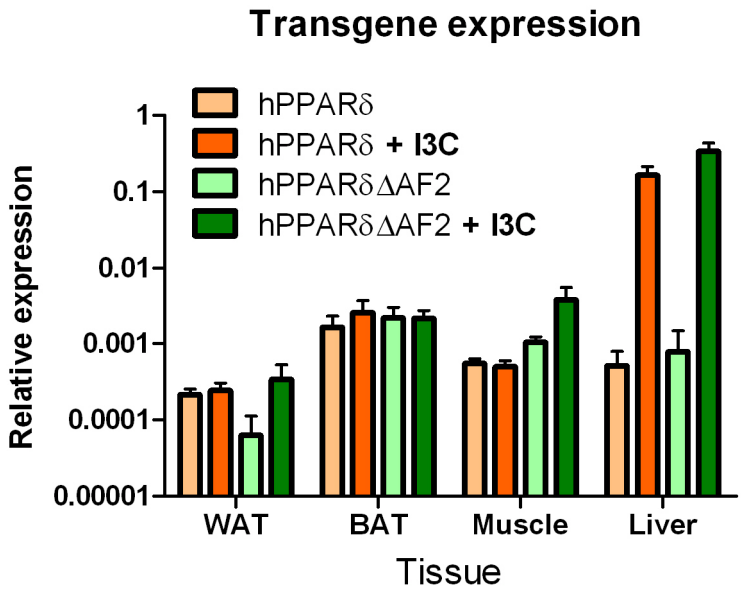

Figure 5.7 Transgene expression in white adipose tissue (WAT), brown adipose tissue (BAT), muscle and liver in transgenic mice fed plain HDF or HFD + 0.25% I3C. n= 6 mice/group.

Surprisingly, in lean animals over-expressing hPPARδ, hepatic gene expression after 18 weeks did not show obvious signs of increased fatty acid oxidation. For

example: CPT1 and Acox1 expression in hPPARδ animals was lower in mice fed HFD+I3C, than in their control group (Fig 5.8 A and B). Whereas FAS mRNA levels had lower values in non-transgenic and hPPARδ mice in groups fed HFD+I3C. It could be concluded that I3C has FAS-inhibiting properties, however, in hPPARδΔAF2 fed HFD+I3C FAS levels were actually higher than in their control groups eating plain HFD (Fig 5.8 C).

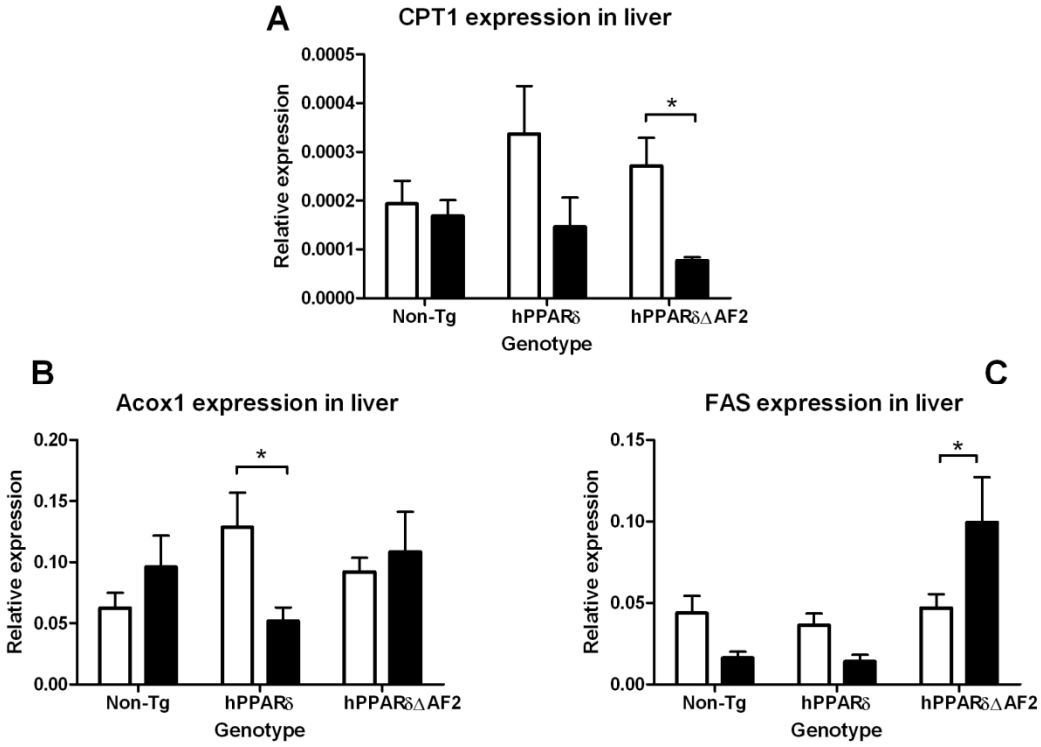

Figure 5.8 Expression of CPT1 (A), Acox1 (B) and FAS (C) in non-transgenic, hPPARδ and hPPARδΔAF2 mice fed HFD (white bars) or HFD+I3C (black bars) for 18 weeks. Two-way ANOVA, significance is indicated as (*p<0.05), n=6 mice/group. Data are expressed as means ± SEM.

Liver PPARδ and PPARγ expression did not reveal any major changes in expression across the genotypes or treatment groups (Fig 5.9 B and C). Unlike

these 2 mentioned members of PPAR family, the third, PPARα was significantly down-regulated in hPPARδ mice fed HFD+I3C and when compared to control and considerably up-regulated in hPPARδΔAF2 animals fed HFD+I3C comparing to their plain HFD fed controls (Fig 5.9 A).

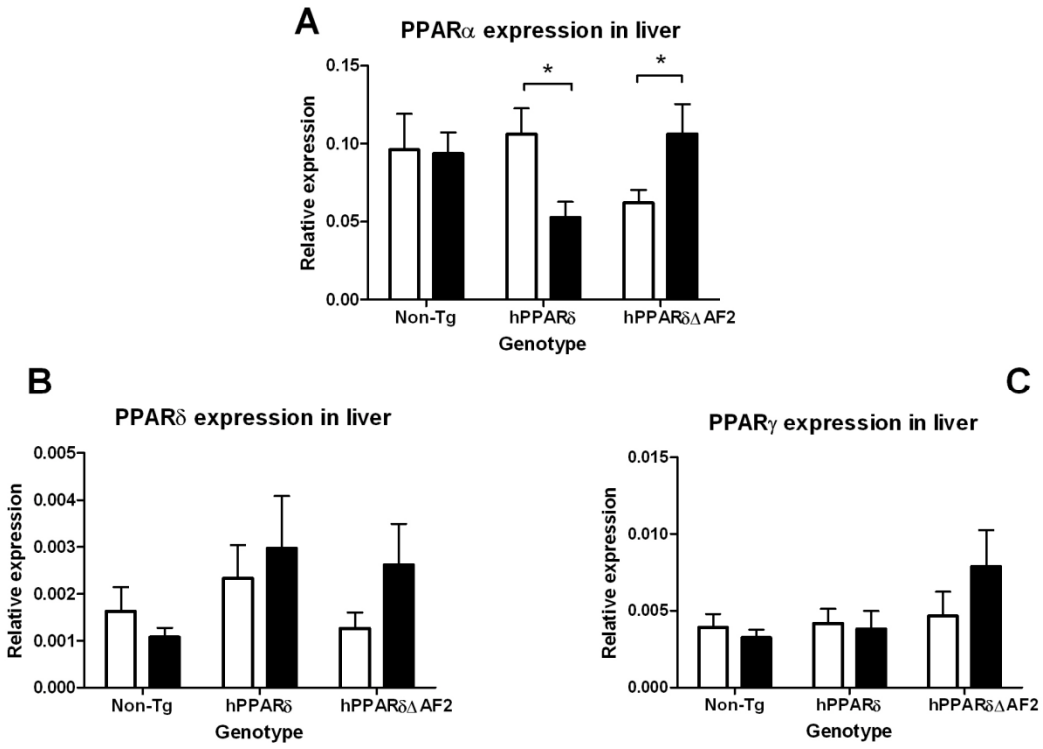

Figure 5.9 PPARs expression in liver in non-transgenic, hPPARδ and hPPARδΔAF2 mice fed HFD (white bars) or HFD+I3C (black bars) for 18 weeks. Two-way ANOVA, significance is indicated as (*$p<0.05$), n=6 mice/group. Data are expressed as means ± SEM.

Genes traditionally accepted as direct or not indirect PPARδ targets like HMGCoAs or ADRP in hPPARδ mice showed down-regulation in mice fed HFD+I3C rather than increasing their levels (Fig 5.10 A and C). ADRP expression reflected the liver triglyceride content rather than being quantitatively responsive to hPPARδ over-expression. On the other hand, such genes like Apoc3 and PDK4, in previous chapter reported to be responsive to PPARδ

activation, in wild type or hPPARδ mice conditionally expressing transgene were not responsive to HFD-activated hPPARδ (5.10 B and D). This was in contrast to mice with induced hPPARδΔAF2 transgene, which have shown several fold increase in mRNA abundance of these two genes over their controls fed pure HFD (Fig 5.10).

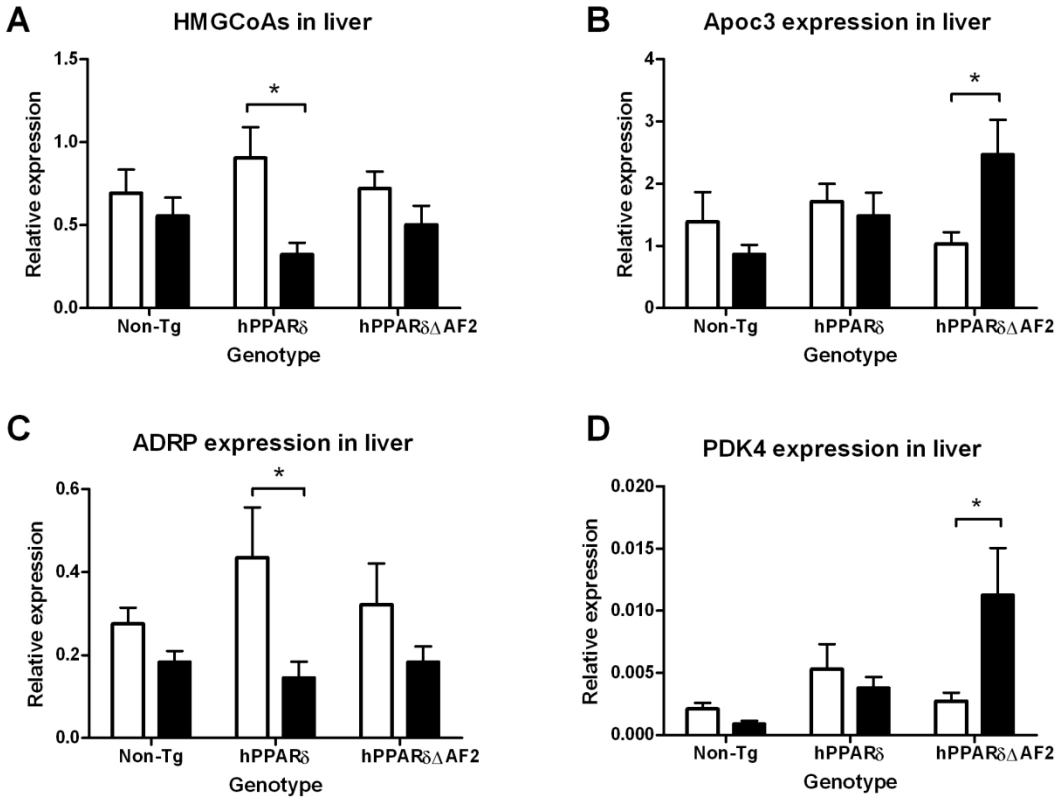

Figure 5.10 Liver PPARδ target genes expression in non-transgenic, hPPARδ and hPPARδΔAF2 mice fed HFD (white bars) or HFD+I3C (black bars) for 18 weeks. Two-way ANOVA, significance is indicated as (*$p<0.05$), n=6 mice/group. Data are expressed as means ± SEM.

Expression of PPARδ genes such as ANGPTL4, PDK4, UCP1, UCP2 or UCP3 and Acox1 in white and brown adipose tissue did not show any statistical

difference between groups or genotypes with the exception of PDK4 and UCP2 in WAT where these two genes were several fold up-regulated in control hPPARδ group (data not shown). However, hPPARδ mice fed HFD+I3C had comparable levels of expression of these genes to the non-transgenic and hPPARδΔAF2 groups.

5.3 Discussion

In previous chapter, mice conditionally over-expressing hPPARδ, when fed diet supplemented with GW501516 demonstrated rapid weight loss and subsequent hepatic steatosis, which was reversed in mice over-expressing dominant negative hPPARδ treated with GW501516. In this chapter's study the GW501516 was eliminated from chow in favour of high fat diet (HFD). Lack of GW501516 in the diet allowed keeping the experiment going for longer than 2 weeks, which before, was usually the maximum time of treatment with GW501516 before this genotype develops psoriasis-like skin disease [117,119].

Over-expression of the hPPARδ transgene prevents from HFD-induced obesity

Feeding the hPPARδ animals with normal RMI diet supplemented with I3C serving as a transgene inducer did not cause weight loss in 2 weeks time (Fig. 4.1A and Fig. 4.2A). In contrast, mice fed HFD+I3C from the first week of experiment started to retard the body growth rate in relation to the mice fed plain HFD. Although I3C has been reported to prevent diet-induced obesity [152], the effects of this compound on weight gain in this study were small and

the effects of modulating hPPARδ activity in these mice was much more substantial (Fig 5.1 B). The effect of transgene induction on body weight gain in hPPARδ mice did not appear to depend on increased food or energy intake, as there were no significant differences observed in food intake among the groups. The only explanation of prevention from increasing the body mass in hPPARδ animals fed diet +I3C was induction of the transgene and subsequent activation of it by dietary fatty acids. Which HFD-derived fatty acids were directly responsible for activation of hPPARδ is unknown, however, 13.46% of the total ingredients of the HFD were composed of monounsaturated fatty acids, which were identified as PPARδ regulators [154]. One of the fatty acids present in HFD was linolenic acid. Although it only contributed up to 0.18% of the total HFD composition used in this study, it was reported before as an efficient activator of the PPARδ, but not PPARα or PPARγ [157]. The more challenging dilemma is the fact that hPPARδΔAF2 mice fed HFD+I3C had significantly retarded body mass gain, when compared to their genetic counterparts fed plain HFD or to the non-transgenic mice fed HFD+I3C. In previous chapter, it was shown that hPPARδΔAF2 animals fed normal chow + I3C in 8 weeks had noticeable but not statistically relevant lower body mass gain rate when compared to non-transgenic mice fed normal chow + I3C. In this experiment HFD makes the difference significant. If the PPARδ function when not activated is in large part repression, then over-expression of dominant negative hPPARδ, possibly relives the repression of certain set of the target genes. It could be possible that group of these gene is responsible for observed retarded body weight gain in hPPARδΔAF2 with the HFD lipids being not efficient enough to bring back the full repression of the hPPARδΔAF2 as GW501516 is capable of,

which was found in previous chapter's study. Nevertheless, the relieving of repression of PPARδ target genes by negative dominance of human version of the receptor is not the equivalent to of the over-expression and activation of the full-length receptor. The weight gain between hPPARδ and hPPARδΔAF2 fed HFD+I3C mice was still significantly different, with the first being almost completely zero (Fig 5.1 E). It is interesting that HFD-induced obesity in non-transgenic animals (Fig 5.1A) was completely abolished in mice over-expressing hPPARδ. What is even more striking is that effect is mainly due to the transgene being expressed in the liver and not in WAT, BAT or muscle. Still, hPPARδ transcriptional activity in the liver has important systemic implications. The pictures showing post-mortem ventral views of hPPARδ mice fed HFD or HFD+I3C clearly indicate that WAT was largely mobilized upon hPPARδ hepatic over-expression.

Effect of over-expression of the transgene is not a direct equivalent of GW501516 signalling

Although PPARδ activity was not directly reflected in a modulation of plasma lipids, where were no obvious distinctions between treatments groups or genotypes, the liver fat was found to be evidently dependent on I3C availability interacting with genotype. After 18 weeks of study the low levels of hepatic triglyceride were found in parallel to induction of the hPPARδ and to lesser extent, to over-expression of hPPARδΔAF2. This finding is consistent with data from studying GW501516 effects in non-transgenic animals treated with the agonist for 8 weeks described in chapter 3 and 4, where the chronic dietary supplementation of GW501516 turned out to be protective against diet-induced hepatic liver accumulation. It is also in accordance with studies performed by

Lee et al where authors did not observe signs of fatty liver with GW501516 treatment up to 6 months in wild-type C57BL/6 mice, but they found short term hepatic fat accumulation [124]. The non-transgenic control mice also clearly show that the protection against HFD-induced liver steatosis in not due to I3C activity (Fig 5.6 A). Unlike the triglyceride, the liver cholesterol presence seems to be promoted by hPPARδ over-expression. However, this effect is entirely gender specific with females the main contributors to the hepatic cholesterol accumulation effect. In studies performed by Meng et al and Auborn et al authors found that indole 3 carbinol used in our study as a transgene inducer is a negative regulator of oestrogen [158,159]. And Gao et al have shown that genetic ablation of oestrogen slulfotransferase, responsible for inactivation of estrogens, decreases several fold liver total cholesterol in female *ob/ob* mice [160]. Therefore the female-specific increase in hepatic cholesterol could be due to impaired oestrogen signalling linked with hPPARδ over-expression.

Taking into account that hPPARδ over-expression had such a profound effect on prevention of diet-induced obesity, it is rather surprising that this metabolic state was not reflected noticeably in hepatic gene expression. In fact, CPT1 and Acox 1 markers of fatty acid oxidation indicated a lower level of fat oxidation when compared with plain HFD controls. It is possible that these 2 markers were reflecting simply the availability of substrate for fatty acid oxidation. That seems to be confirmed by PPARα mRNA transcripts which were found to be less abundant in mice over-expressing hPPARδ. On the other hand mice conditionally expressing hPPARδΔAF2 had elevated levels of PPARα mRNA, which is consistent with the hepatic PPARα mRNA expression data in hPPARδΔAF2 fed normal diet + I3C, shown in previous chapter. Traditionally

accepted PPARδ target genes like ADRP, HMGCoAs or PDK4, also shown down-regulation or turned out to be non-reactive to hPPARδ over-expression in presence of HFD. This might indicate a limited availability of substrate for β-oxidation as already mentioned.

In summary HFD feeding promotes diet-induced obesity and hepatic lipid accumulation in C57BL/6 wild type mice. These effects however were tempered efficiently by long term over-expression of hPPARδ in the liver. In the two previous chapters, we presented evidence for PPARδ-promoted hepatic accumulation in wild-type and transgenic mice. The mechanism of clearance of liver fat in long-term period in study involving wild-type animals and possibly in animals used in this chapter's experiments needs further investigation. The next chapter aims to address this.

Chapter 6 Effect of genetic ablation of PPARα or PPARδ on PPARδ-driven hepatic lipid accumulation. Study of PPARα-KO and PPARδ-KO mice.

6.1 Introduction

As shown in previous chapters, PPARδ pharmacological activation using the potent synthetic ligand GW501516 promotes liver lipid accumulation accompanied by weight loss. This effect is markedly enhanced by conditional over-expression of human PPARδ. However, regardless of stable GW501516 blood levels or hPPARδ over-expression and despite still available peripheral fat deposits hepatic triglyceride disappear in long-term treatment. It might suggest steady build up or activation of a hepatic fat-burning factor. One of the main transcription factors governing fat oxidation is PPARα, abundantly expressed in liver. Recent evidence [17] shows identification of an endogenous PPARα ligand, which production as the authors suggest, is dependent on fatty acid synthase activity. Generation of this ligand was suggested by Barroso et al as an explanation of the beneficial effect of GW501516 in lowering plasma triglyceride in HFD-fed mice [161]. On the other hand, Terada et al shows some evidence that GW501516 can be efficient PPARα activator. The authors of this study speculated on whether the GW501516 is a direct activator of PPARα or that it may activate PPARα through an indirect mechanism such as by the production of an endogenous ligand of PPARα [162].

While our results using the PPARδ transgenic animals confirm a role for PPARδ in the action of GW501516 these experiments do not address the possibility of an additional contribution of PPARα to the process. In order to assess the requirements for PPARα and PPARδ in the action of GW501516 we

used of PPARα knockout (PPARα-KO) and PPARδ knockout mice (PPARδ-KO). PPARα-KO mice were shown to be resistant to fibrate-promoted hepatomegaly, observed in wild type-animals treated with these compounds [95]. However, they are susceptible to fasting-induced fatty liver, showing the important role of PPARα in liver fat oxidation and processing [25,163].

By treating PPARα-KO mice with GW501516 we investigated if PPARα activation and signalling is directly involved in long-term clearance of PPARδ-promoted liver triglyceride accumulation. Additionally, by studying the PPARδ-KO animals fed GW501516, we tested the hypothesis if weight loss and liver steatosis shown in the experiments presented in previous chapters, can be solely attributed to PPARα's cross-reaction with GW501516.

6.2 Results

To first present how PPARα is important in the liver rodent as a major driving force of β-oxidation, here we show the influence of fasting on hepatic lipid response and to lesser extent on plasma lipid profile in wild type and PPARα-KO mice. Thirty 10-15 week old male non-transgenic and PPARα-KO mice (3 per group) were fasted for 0, 6, 16 and 24 hours. Interestingly, after 6 hours of fasting the initial rise in hepatic triglyceride was noticeable in non-transgenic, but not in PPARα-KO animals. However, after 16 and 24 hours of food deprivation the hepatic triglyceride levels in PPARα-KO exceeded those found in the wild type mice by 2.5 and 6.3 fold respectively and this effect was considered as very significant (Fig 6.1).

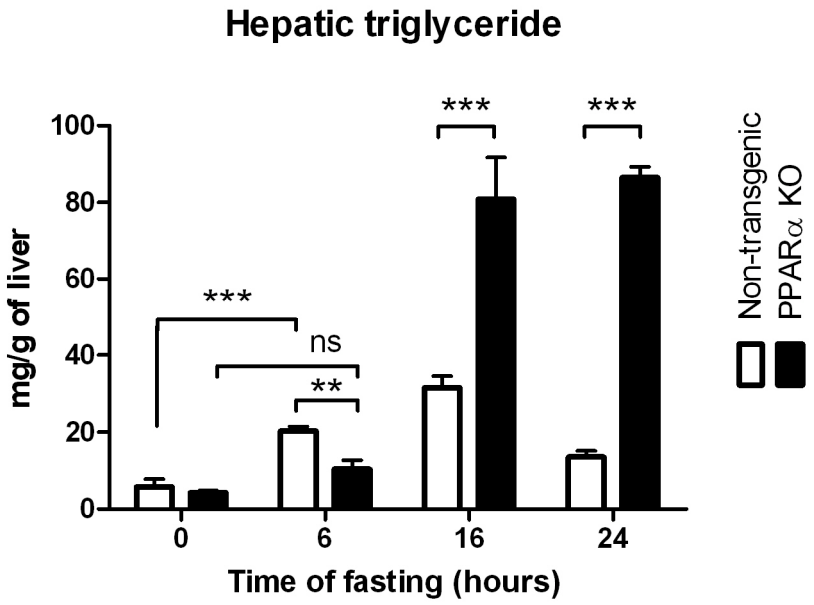

Figure 6.1 Fasting induced hepatic lipid accumulation in non-transgenic and PPARα-KO animals. Two-way ANOVA, significance is indicated as: ** $p<0.01$; *** $p<0.001$, n=3 mice/group. Data are expressed as means ± SEM.

The lipid response in the liver was partly reflected in plasma triglyceride profile, where in PPARα-KO mice blood triglyceride were only significantly elevated after 16 hours of fasting (Fig 6.2 A). On the other hand, a very similar profile of lipid fluctuation was revealed after measurements of free fatty acids (FFA) in plasma, where FFA levels mostly overlapped with hepatic triglyceride. It suggests indirectly, that the source of hepatic fat was blood borne (Fig 6.2 B) as main lipid export from the liver is in lipoprotein conjugated form and not as FFA.

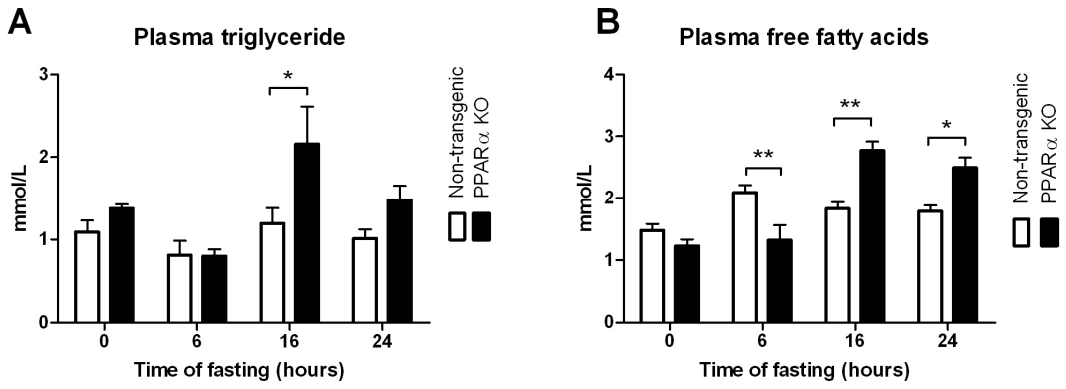

Figure 6.2 Plasma triglyceride (A) and FFA (B) in fasted non-transgenic (white bars) and PPARα-KO mice (black bars). Two-way ANOVA, significance is indicated as: * $p<0.05$ ** $p<0.01$; *** $p<0.001$, n=4 mice/group. Data are expressed as means ± SEM.

After confirming how PPARα is important for processing hepatic lipids during short time food deprivation, we proceeded to examining long-term effect of hepatic lipid reaction to chronic pharmaceutical activation of PPARδ receptor in PPARα-KO animals.

24 10-week old PPARα-KO mice ((B6.129S4-*Ppara*^{tm1Gonz}/J), 2 females, 2 males per group) were placed on control or a diet containing GW501516 (0.0025% w/w). After two weeks, 4 mice (4 from each group) were sacrificed. Then after 1 month from beginning of the experiment a further 8 mice were taken with the remaining mice being sacrificed after 2 months. Before the sacrifice mice were not fasted to avoid interference from the phenotype described above.

Unexpectedly, in the first weeks of feeding mice with the diet supplemented with GW501516, PPARα-KO animals did not show any sign of weight loss (Fig 6.3A) or reduced adiposity when sacrificed after 2 weeks (data not shown), when compared to their controls fed plain chow. What is more,

animals fed a diet enriched with GW501516 after two weeks put a more on weight than their controls and this effect was significant (2.1 fold, P=0.02) (Fig 6.3 A and B).

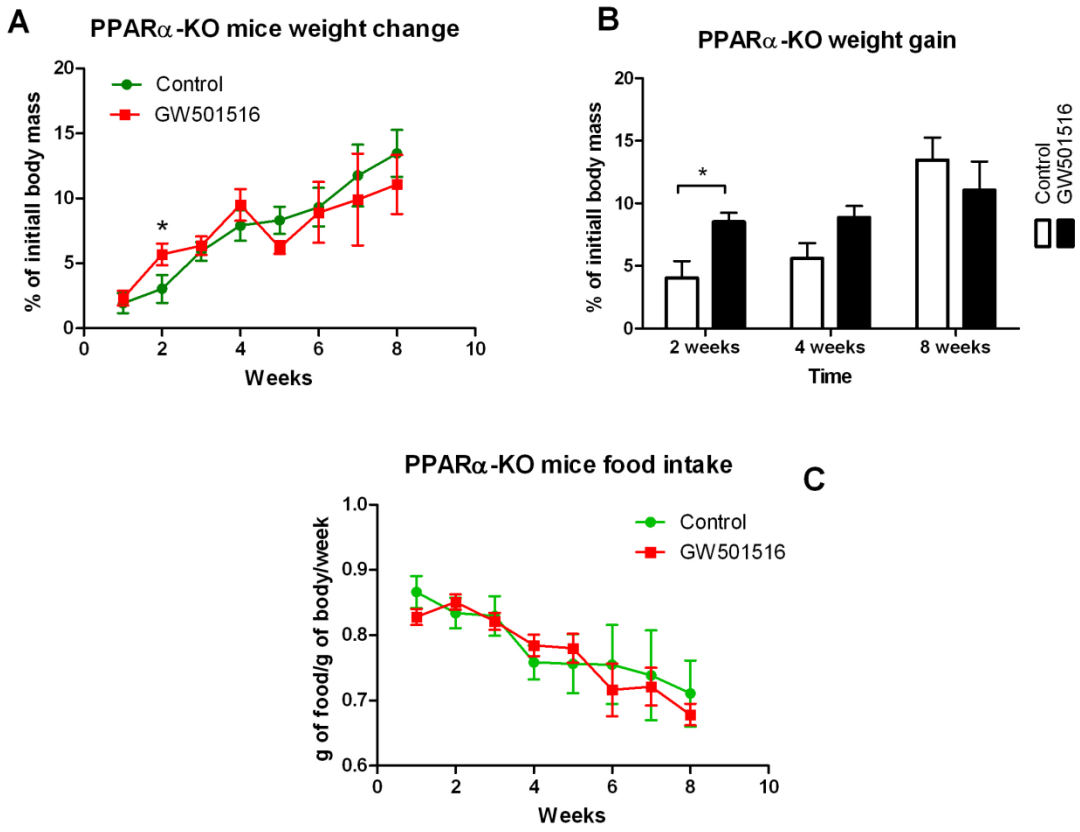

Figure 6.3 Weight gain (A and B) and food intake (C) in PPARα-KO mice fed PPARδ selective agonist GW501516. T-test and two-way ANOVA, significance is indicated as: * $p<0.05$, n=4 mice/group. Data are expressed as means ± SEM.

As might be expected, there was also no difference in food intake between both treatment groups (Fig 6.3 C). The beneficial effect of lowering plasma triglyceride upon GW501516 treatment also disappeared in PPARα-KO mice (Figure 6.3 A), although the correction must be made for measuring non-fasted animals. Plasma total cholesterol had a trend to be noticeably higher in GW501516 treated animals but the overall effect was not significant (Fig 6.3 B).

Despite the lack of functional PPARα, the rise in plasma HDL levels upon GW501516 treatment was still detectable. At 4 and 8 weeks time points HDL levels were higher in treated groups, when compared to controls, however, with only 4 weeks time measurement difference being statistically significant ($P<0.05$) (Figure 6.3 C).

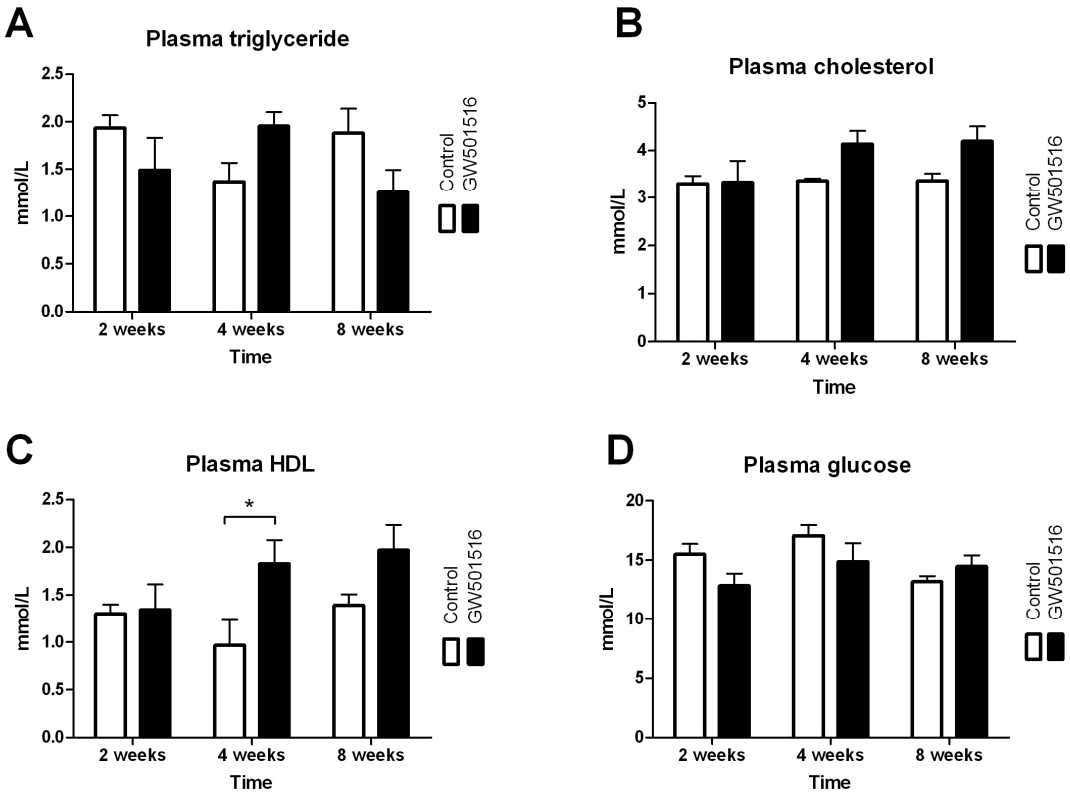

Fig 6.3 Plasma lipids in PPARα-KO mice fed normal or diet supplemented in GW501516. White bars – mice fed normal chow, black bars - animals fed diet enriched in GW501516. Two-way ANOVA, significance is indicated as: * $p<0.05$, n=4 mice/group. Data are expressed as means ± SEM.

As expected with the lack of weight loss in GW501506-treated mice, the levels of plasma insulin and leptin were not modulated by GW501516 in PPARα-KO animals (Fig 6.4), which shows indirect influence of PPARδ ligand

on especially leptin levels as it was seen in wild type animals and animals over-expressing hPPARδ.

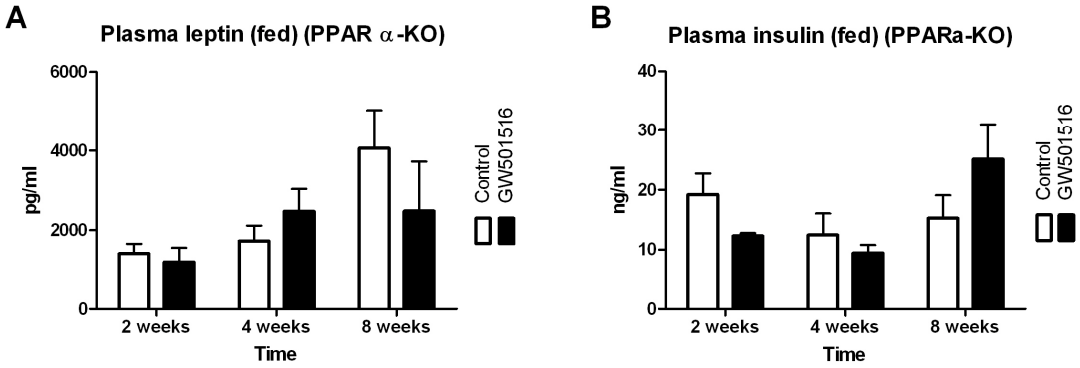

Figure 6.4 Leptin (A) and insulin (B) levels in mice fed normal chow (white bars) or fed diet supplemented with GW501516 (black bars), n=4 mice/group. Data are expressed as means ± SEM.

Measurements of liver triglyceride have revealed no difference in hepatic fat, including total cholesterol between the treatment groups (Fig 6.5). This is in total contrast to the observations found in wild type or in animals transgenic for hPPARδ in previous chapters, where GW501516 had profound role in promoting liver steatosis in these animals.

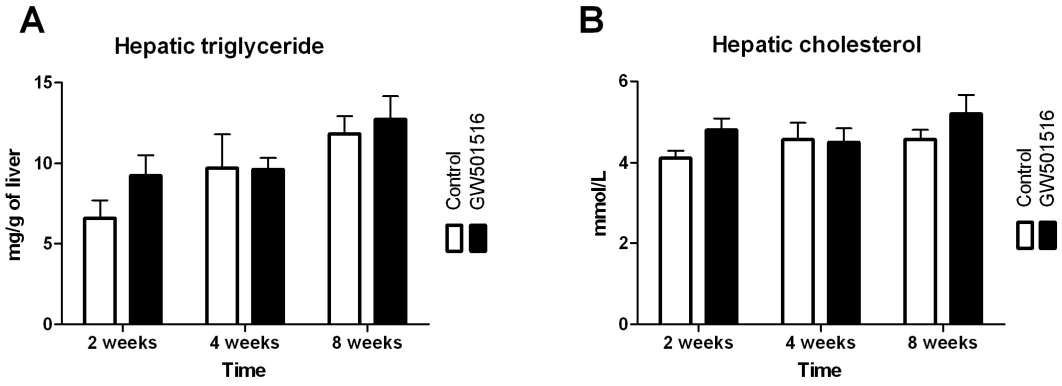

Figure 6.5 Hepatic triglyceride (A) and hepatic cholesterol (B) levels are not affected by GW501516 treatment in PPARα-KO mice. White bars – mice fed normal chow, black

bars - animals fed diet enriched in GW501516, n=4 mice/group. Data are expressed as means ± SEM.

Although a fatty liver phenotype and PPARδ-dependent weight loss were completely abolished in PPARα-KO mice fed PPARδ agonist, gene expression in that group showed that many direct PPARδ target genes were not only still up-regulated, but their expression levels were markedly more pronounced.

For example, liver ADRP mRNA levels were significantly higher at every time point (3 - 5.5 fold) ($P<0.001$) (Figure 6.6 A). Consistent with lack of liver steatosis in GW501516 treated animals, no correlation was found between ADRP and hepatic triglyceride (data not shown). The angiopoietin-related protein 4 (ANGPTL4) is involved in lipid metabolism and is the target of PPARδ. Hepatic ANGPTL4 mRNA levels were also elevated in treatment group 23.5, 2.5 and 6.7 fold (at 2, 4 and 8 weeks respectively) (Figure 6.6 C). Also HMGCoAs and UCP2 have shown evident expression upon GW501516 treatment (Figure 6.6 B and D).

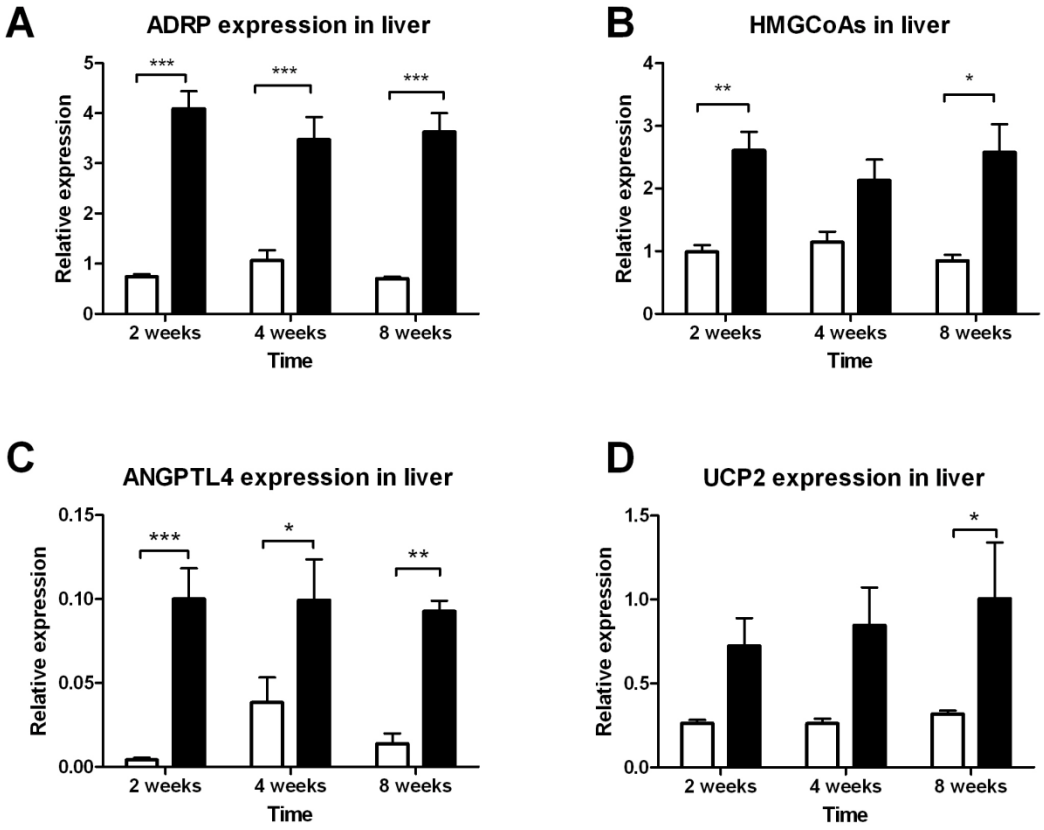

Figure 6.6 Gene expression pattern of PPARδ target genes in livers of PPARα-KO mice fed normal chow (white bars) or diet supplemented in GW501516 (black bars). Two-way ANOVA, significance is indicated as: * $p<0.05$; ** $p<0.01$; *** $p<0.001$, n=4 mice/group. Data are expressed as means ± SEM.

In addition, muscle mRNA levels of pyruvate dehydrogenase kinase isozyme 4 (PDK4) (inhibition of carbohydrate metabolism), ANGPTL4 (lipid metabolism), UCP2 and UCP3 (proteins uncoupling) were profoundly increased in mice fed diet enriched in GW501516. All of them are PPARδ target genes (Fig 6.7 A-D).

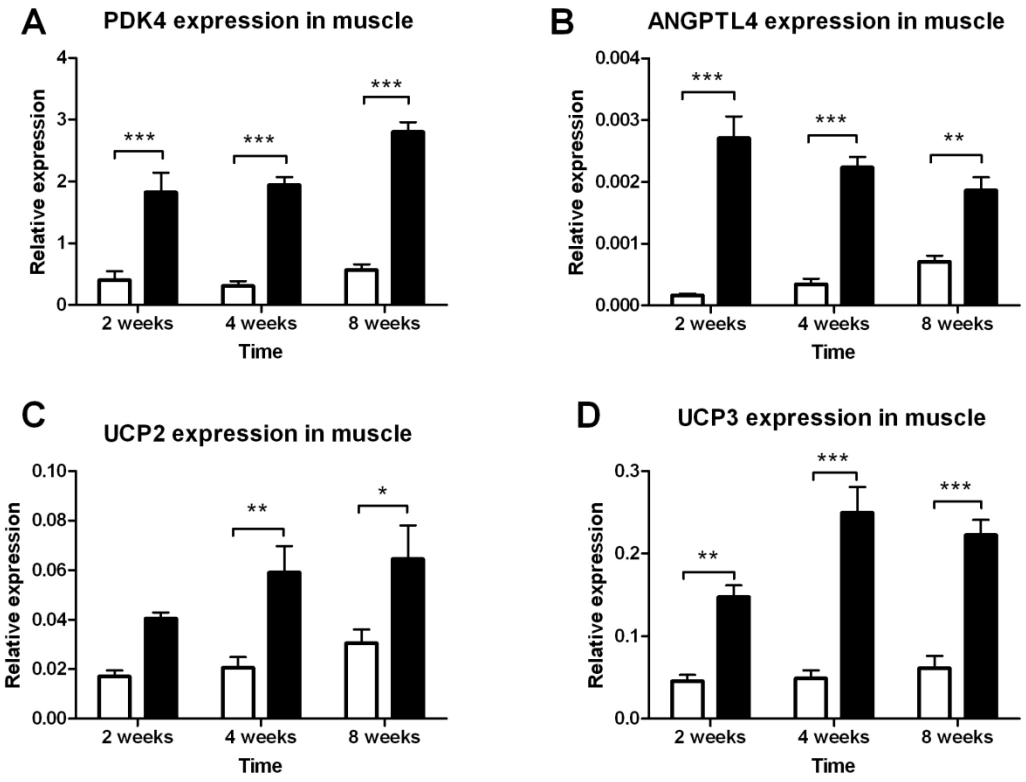

Figure 6.7 Expression of PPARδ target genes in muscle of PAPRα-KO mice fed normal chow (white bars) or diet supplemented in GW501516 (black bars). Two-way ANOVA, significance is indicated as: * $p<0.05$; ** $p<0.01$; *** $p<0.001$, n=4 mice/group. Data are expressed as means ± SEM.

In previous chapters, genes such as CPT1, Acox1 Apco3 were found to be significantly dependent on GW501516 treatment. In PPARα-KO animals however, excluding the case of Acox1, which have revealed some trend rising trend in presence of PPARδ agonist, the overall hepatic expression of these genes were not significantly dependent on GW501516 availability (Fig 6.8).

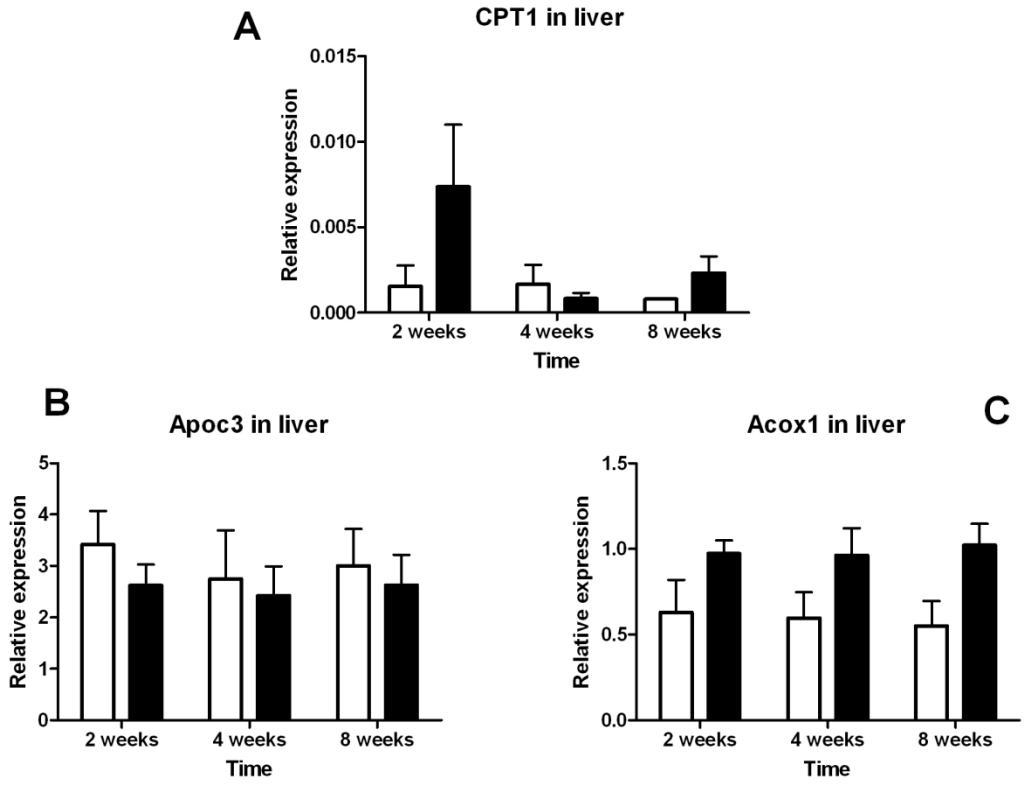

Figure 6.8 CPT1 (A), Apoc3 (B) and Acox1 (C) expression in livers of PPARα-KO mice fed normal chow (white bars) or diet supplemented in GW501516 (black bars), n=4 mice/group. Data are expressed as means ± SEM.

FAS mRNA levels, in general, turned out to be non-reactive to PPARδ agonist in PPARα-KO mice. Only muscle after 4 weeks treatment have shown noticeable increase in mRNA abundance in GW501516 treated animals (Fig. 6.9).

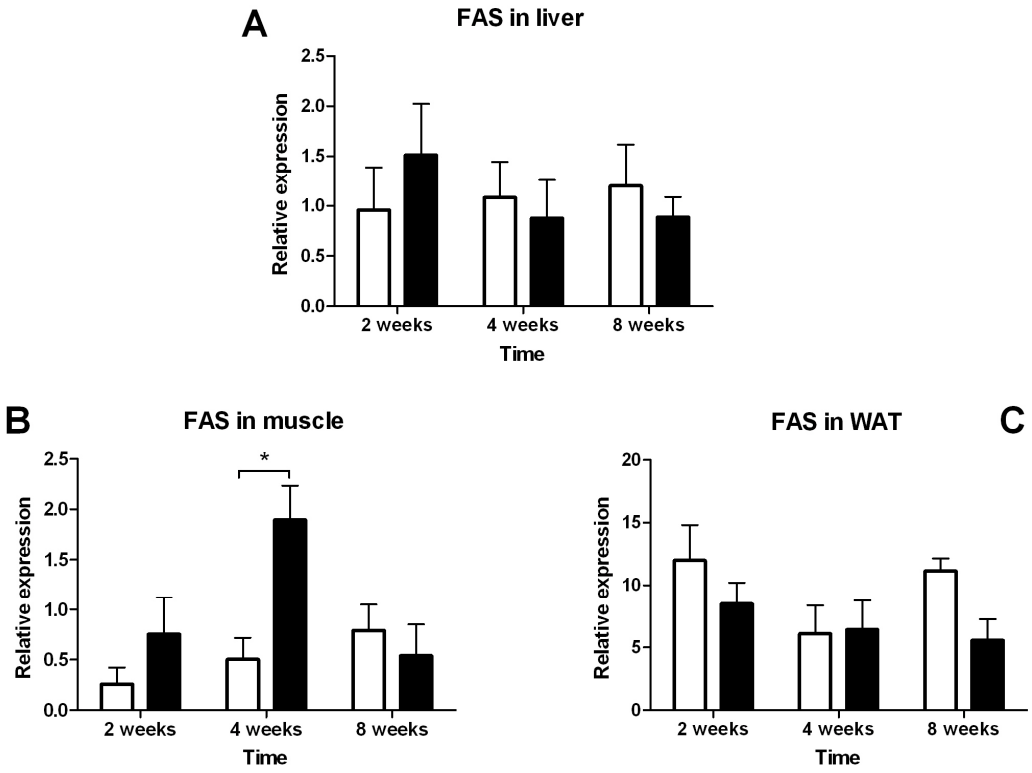

Figure 6.9 Fatty acid synthase levels in liver (A), muscle (B) and white adipose tissue (C) of PPARα-KO mice fed normal chow (white bars) or diet supplemented in GW501516 (black bars). Two-way ANOVA, significance is indicated as: * $p<0.05$, n=4 mice/group. Data are expressed as means ± SEM.

Although in the PPARα-deficient mice we used in this study the PPARα mRNA is still expressed, the transcript is aberrant and no functional protein is made [77]. Therefore only PPARδ and PPARγ mRNAs expression levels were screened. Interestingly, PPARα ablation caused observable trend in decrease of PPARδ mRNA levels in WAT and muscle in mice fed diet supplemented with GW501516, although only some time points have reached statistical significance. PPARγ expression showed no signs of variability between treatments groups in liver and muscle, but in muscle the tendency to drop of mRNA level of this PPAR family member in WAT was noticeable (Fig 6.10).

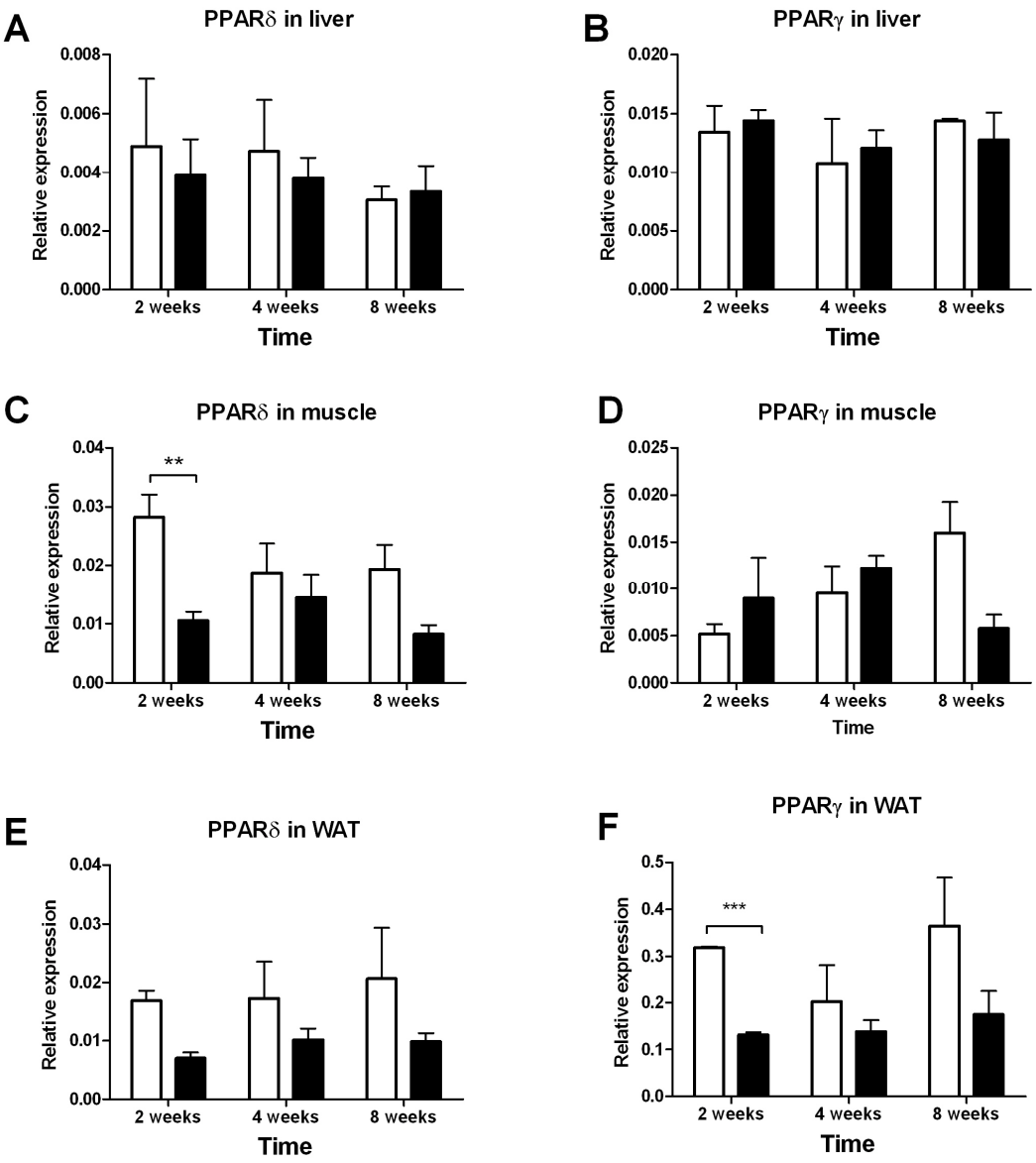

Figure 6.10 PPARδ and PPARγ expression in liver, muscle and WAT of PPARα-KO mice fed normal chow (white bars) or diet supplemented in GW501516 (black bars). Two-way ANOVA, significance is indicated as: ** $p<0.01$; *** $p<0.001$, n=4 mice/group. Data are expressed as means ± SEM.

A similar experiment was conducted using PPARδ knockout animals. For two weeks time C57/BL6 PPARδ-KO male and female mice (5 mice per group)

were fed normal chow or diet enriched in 0.0025% GW501516 (w/w). In this time, PPARδ agonist treatment did not cause weight loss when compared to control group (Fig 6.11 A), neither changed food intake, which was typical to the previous experiments conducted in wild-type animals or conditionally-expressing hPPARδ (Figure 6.11 B).

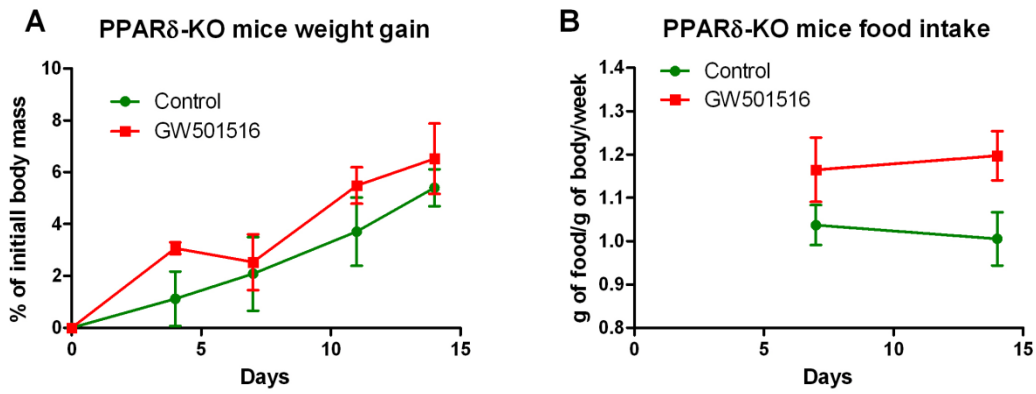

Figure 6.11 Weight gain of PPARδ-KO mice fed normal chow and diet supplemented with GW501516 in 2 weeks. n=5 mice/group

No differences were found in hepatic lipid content between both groups (P=0.203) and in hepatic cholesterol (Figure 6.12A). PPARδ-KO mice turned out to be non-responsive to GW501516, therefore the experiment was discontinued.

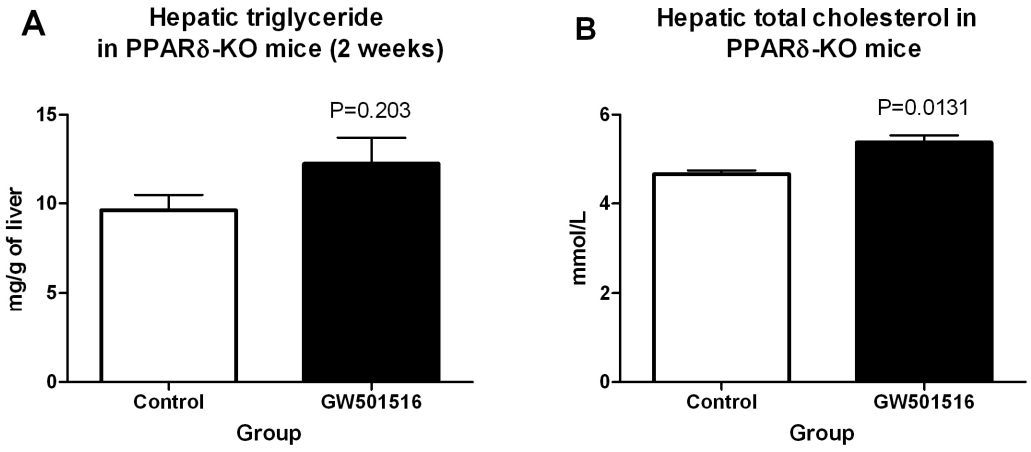

Figure 6.12 Hepatic steatosis was not present in PPARδ-KO mice fed GW501516 in two weeks although total liver cholesterol difference between treatment groups reached significance, n=5 mice/group. Data are expressed as means ± SEM.

With the available liver lipids specimens from PPARδ-KO mice and the proposed explanation of PPARα involvement of long-term clearance of hepatic fat, the hypothesis that PPARδ activation leads to steady build-up of endogenous PPARα ligand now could be tested. Hepatic lipids from PPARδ-KO mice were compared with lipids extracted from wild-type animals fed GW501516 from the experiment described in chapter 5. The proposed PPARα endogenous activator: 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) [17] was detected in whole hepatocyte lipid extracts using LC-MS analysis. Basal hepatic levels of POPC from 2 weeks time point in PPARδ-KO were significantly higher than in non-transgenic mice (Fig 6.13). The time was also a key factor in increasing levels of POPC in livers of animals fed diet enriched in GW501516 throughout the length of the study, where the difference within the treatment groups between 2 versus and 4 and 8 weeks was 3 fold in favour of the latter ones ($P=0.0065$ and $P<0.001$ respectively). This data shows

that disappearance of hepatic lipids seen in GW501516 treated groups between 4 and 8 weeks in non-transgenic animals (Figure 3.5) co-exists with accumulation of POPC over the same time period, supporting the hypothesis that this is a *bona fide* endogenous PPARα ligand that may provide the downstream activation of PPARα in response to activation of PPARδ.

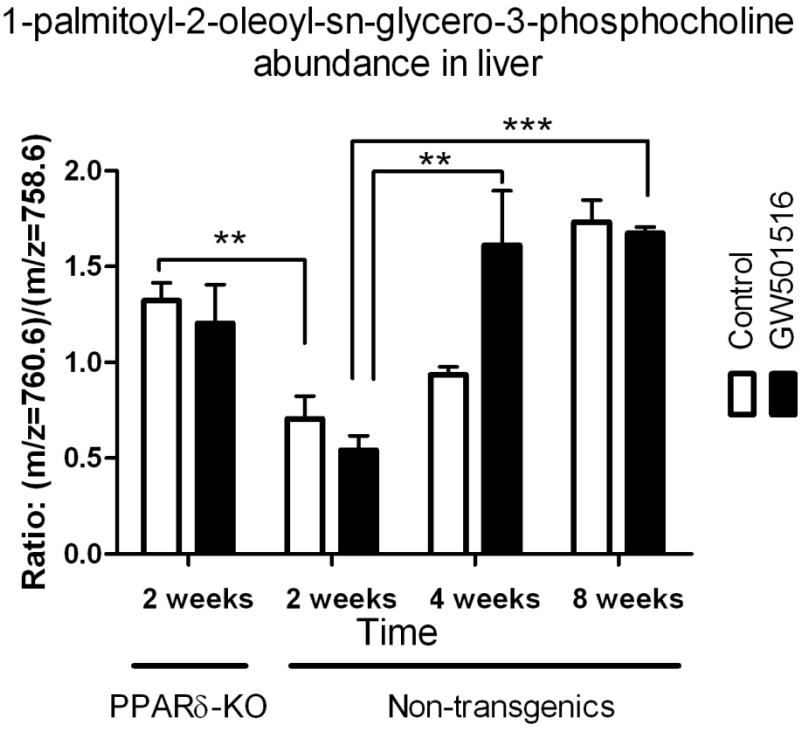

Figure 6.13 Endogenous PPARα ligand abundance in livers of PPARδ-KO mice and non-transgenic mice fed normal chow or diet supplemented with GW501516. T-test and two-way ANOVA, significance is indicated as: ** $p<0.01$, ***$p<0.001$, n=4 mice/group. Data are expressed as means ± SEM.

6.3 Discussion

This chapter's main objectives were to investigate impact of null mutations on both PPARα and PPARδ on the physiological actions of GW501516. In previous chapters we had demonstrated that modulation of PPARδ activity profoundly affected response to GW501516, however other studies have suggested a role for PPARα in the response to this drug, and our results had suggested a potential temporal interplay between these two receptors in hepatic lipid regulation.

PPARα is involved in modulation of body mass in mice

The main surprising finding was that PPARα-KO animals did not lose weight in response to GW501516, demonstrating an absolute requirement for this receptor in the weight loss response to this drug.

We confirmed the profound role for PPARα in the mobilization of adipose tissue deposits upon fasting. The knockout mice displayed an exaggerated fasting response after 16 hours with increases in plasma levels of FFA and liver triglyceride, as has been previously published. However, elevated plasma free fatty acids and hepatic lipid accumulation was evident in wild-type animals after only 6 hours of fasting, but not in PPARα-KO animals. The mobilization of peripheral fat deposits was evidently delayed in KO animals. This is a novel and surprising observation. It suggests a role for PPARα not only in governing the genes involved in oxidation of fatty acids but also in controlling the rate of release of fatty acids from adipose cell. Hormone sensitive lipase (HSL) one of the "gatekeepers" of lipolysis is activated predominantly by phosphorylation, triggered by low insulin levels or catecholamines and is active in low blood

glucose state, which is typical to fasting. It is a rate-limiting enzyme for adipose triglyceride lipolysis [15]. The observed delayed release of FFA in PPARα-KO mice might be due to possible PPARα participation in controlling this process. One of the first indications that PPARα activity might include involvement in adipose tissue lipid stores mobilization was work done by Perreault et al. In his study he showed that in the absence of PPARα, the PPAR pan agonist (compound 4) was not able to induce drug-promoted weight reduction [144]. However, the author's conclusion was that compound 4 treatment weight reduction capabilities come mainly from the fact of suppressed appetite, which is provoked by intermediates generated during oxidation of fatty acids, which then regulate food intake in treated mice, and not by stimulating lipolysis. Indeed, the PPAR agonist has the potential to reduce the appetite, as it was shown in experiment in wild-type animals fed GW501516 described in chapter 3, however, mice conditionally over-expressing hPPARδ and fed diet enriched in GW501516 did not show diminished appetite, but in the same time lost significant proportion of total body weight (chapter 4).

PPARα and PPARδ co-ordinately modulate body weight gain

The role of PPARα in mobilizing adipocyte fat stores is becoming more evident when analyzing action of PPARδ selective ligand in PPARα-KO mice. These mice fed GW501516 did not exhibit any weight loss in spite of massive up-regulation of the PPARδ target genes involved in lipid oxidation like UCP2 and UCP3, genes involved in generation of ketone bodies like HMGCoAs or inhibitors of carbohydrate metabolism like PDK4. The fact that GW501516 was inefficient in causing weighs loss in PPAR-KO mice was not due to absence of

up-regulation of the genes involved of whole process of lipid utilization. It was rather the result of failure of the PPARα-KO mice to mobilize its peripheral white adipose tissue stores in presence of PPARδ ligand. Similar dependence of PPARδ activity on PPARα presence and signalling was revealed in study done by Faiola et al. In their work, authors have found that increases in liver weights and clinical chemistry indicators of skeletal muscle damage and/or liver injury were more pronounced in wild-type mice compared with PPARα-KO mice administered toxicological doses of selective PPARδ agonist GW0742 [164]. However, they concluded that absence of PPARα eliminates the cross-activation of this receptor with PPARδ ligand. Similar hypothesis was constructed by Terada et al, where he found that the effect of GW501516 on several PPAR target genes in primary hepatocytes and the *in vivo* liver studies depends on the expression of PPARα. The conclusions was that that activation of PPARα by GW501516 can be explained by two mechanisms: either by direct activation by binding of GW501516 to PPARα or by indirect activation through direct binding and activation of PPARδ [162]. Our experiment with PPARδ-KO mice shows that theoretical cross-activation of GW501516 with PPARα is not sufficient to induce body mass reduction through peripheral fat mobilization. This is the condition as we assumed based on experiments shown in previous chapters, for the major cause of observed liver fat accumulation observed in non-transgenic and especially in mice over-expressing hPPARδ. In Terada et al study the authors however, do not show phenotypic observation of GW501516 action in PPARα-KO mice, focusing rather solely on transcriptional regulation patterns. Nevertheless, there are some important differences in their finding and our observations. For example we have shown that PDK4 is still up-regulated in

muscle of PPARα-KO mice in the presence of GW501516 (Fig 6.7 A) and PDK4 expression in liver shows trend for up-regulation after treatment with GW501516, although it was not statistically significant (data not shown). However, in Terada et al work, authors present data showing down-regulation of this gene in livers of PPARα-KO mice fed diet enriched in GW501516.

PPARδ activation leads to generation of PPARα endogenous ligand

Consistent conclusion from studies performed by Terada et al and Barroso et al and also from our work was that PPARδ pharmacological activation might lead to generation of endogenous PPARα ligand. So far only Barroso et al have presented some evidence supporting this hypothesis, where they have shown that mice fed high fat diet and treated with GW501516 had elevated hepatic levels of 16:0/18:1- phosphocholine, previously identified as endogenous PPARα ligand [17], when compared to mice fed normal chow or plain HFD [161]. Here we present that generation of this ligand is dependent on presence of functional PPARδ or related to GW501516 treatment and time dependent (Fig 6.13). The elevated levels of this phosphocholine molecule in PPARδ-KO mice are consistent with the hypothesis that physiologically un-liganded PPARδ works as a repressor and with ligand binding or genetic ablation of the receptor the repression is relieved and sets of target genes is up-regulated or down-regulated [148,153]. It is possible that among these genes, are the one involved in process of generation of 16:0/18:1- phosphocholine specimens. The original work of Chakravarthy et al associating this particular phosphocholine specimen with endogenous PPARα ligand suggests strong involvement of fatty acid synthase in production of this molecule. Unlike their

findings, our work only shows evidence for up-regulation of FAS in the livers of wild-type animals at the latest stage of experiment (Fig 3.9). Our data would suggest that PPARδ modulates the production of POPC via an alternative pathway other than through FAS activity, although the nature of this remains unknown.

PPARδ activity promotes white adipose tissue lipolysis

The steady build up of PPARα ligand through chronic PPARδ activation does not however explain the inability of PPARα-KO animals to mobilize their white fat peripheral deposits in the presence of GW501516, so well documented in non-transgenic and mice over-expressing hPPARδ in previous chapters. Moreover, the exact mechanism how pharmacological PPARδ activation leads to adipose tissue lipolysis is still not well deciphered. Some evidence was provided by Staiger et al, where in their work they have shown that plasma ANGPTL4 positively correlated with fasting free fatty acids and adipose tissue lipolysis [165]. ANGPTL4 is well known PPARδ target gene and other studies like one performed by Gray et al report it as a crucial element of lipolysis induction [166]. The authors of the latter study place ANGPTL4 function as crucial for fasting, glucocorticoids, and catecholamines stimulated triacylglycerol hydrolysis in murine fat. Fasting lowers the insulin levels, which is known HSL repressor [15] and together with glucocorticoids, and catecholamines leads it to activation of HSL. Still, these conditions are not met in pharmacological induction of adipocyte lipolysis and subsequent weight reduction observed in wild-type mice treated with GW501516 or hPPARδ over-expressors. These mice were not exercised or semi-starved and after sacrifice the insulin levels

were not different between treatment groups. Additionally, in the study performed by Narkar et al there was no increase in HSL transcripts in the quadriceps of GW501516 treated mice, although they have shown that co-treatment with AICAR, the AMP-activated protein kinase (AMPK) agonist, does induce HSL transcription in muscle [121]. The potential explanation how the fat is mobilised by PPARδ activity without or with minimal induction of HSL, could lie in activation of another lipase present in WAT – adipose triglyceride lipase (ATGL) [167]. ATGL is now considered to be the major triglyceride lipase in adipose tissue, hydrolyzing triacylglycerol to diacylglycerol and a FFA [168]. The increase of transcripts of ATGL, were found in Staiger et al study, where C2C12 myocytes were treated with GW501516. They have also shown the mRNA of ATGL was co-expressed with ANGPTL4 transcripts in the same cell line. Additionally, recent report from Fuchs et al shows that ATGL knockout mice have lower levels of hepatic endoplasmic reticulum stress compared to wild-type mice, which is typical in non-alcoholic fatty liver disease [169]. Also the work done by Mandard et al shows that over-expression of ANGPTL4 in peripheral tissues increases significantly levels of ATGL, but not HSL, in adipocytes [170]. Taken together, the importance of secretory protein ANGPTL4 and ATGL activation might indicate the right direction how pharmacological and in particular hepatic PPARδ activation promotes WAT lipolysis. However, this effect is completely abolished in PPARα-KO mice despite massive up-regulation of ANGPTL4 mRNA in muscle and liver upon GW501516 treatment (Fig 6.6C and 6.7B respectively). Evidently, PPARα presence is essential for induction of triglyceride hydrolysis by PPARδ pathway. Most likely through preventing ATGL induction and/or activation, but not through HSL, which is still active in PPARα-

KO animals because it was shown they are still prone to fasting-induced fatty liver. The delayed appearance of elevated plasma FFA in fasted PPARα-KO mice seems to support this hypothesis as the ATGL is the lipase, which first hydrolyses triacylglycerol to diacylglycerol and FFA [171]. Summarizing, genetic PPARα knockout deprives the mouse body an important factor, which enables PPARδ agonist-dependent weight loss and subsequent liver lipid accumulation. Equally probable is also that ablation of PPARα frees the potential repressor-like factor, which interferes with PPARδ-related WAT lipolysis, in normal condition repressed by PPARα presence. Such pattern of regulation was presented by Adhikary et al, where they have shown that inhibition of PPARδ transcripts using siRNA, de-represses defined set of genes.

Therefore, necessity arises to explore and expose the complex transcriptional relationship between PPARδ and PPARα in relation to regulation of body weight and hepatic lipid accumulation by these receptors. In the next chapter we have assessed genome-wide transcriptional profiles of GW501516 effects in the livers of various mouse genetic models, in order to provide some overview of these complex interactions.

Chapter 7 Genome-wide transcriptional profiling of PPARα and PPARδ cooperation in regulation of gene expression.

7.1 Introduction

The evidence from previous chapters shows that signalling from both PPARα and PPARδ is crucial for GW501516-induced physiological effects such as white adipose tissue lipolysis and subsequently weight loss and hepatic triglyceride accumulation. There are other recent reports on close cooperation between orphan receptors. For example, nuclear receptors Rev-erbα and Rev-erbβ regulate circadian rhythm and metabolism. Depletion of both Rev-erbα and Rev-erbβ causes marked hepatic steatosis, in contrast to relatively subtle changes upon loss of either subtype alone [172]. As previously discussed Adhikary et al proposed several modes of transcriptional regulation by PPARδ. One is the classical ligand-dependent relief of repression of target genes. Two additional patterns of transcriptional regulation described in that work revealed that PPARδ presence is required for stable expression of a defined set of genes and second is that siRNA-mediated depletion of PPARδ up-regulates groups of genes, which are normally ligand non-responsive [153]. If we add another factor to the equation like presence or absence of PPARα, the situation can complicate even further as the results from previous chapter suggest. Furthermore, in our published work we have shown that expression of both hPPARδ and hPPARδΔAF2 abolish bezafibrate-induced hepatomegaly and PPARα target genes expression in liver [140]. With the availability of the multiple genetic mice models (wild type, hPPARδ, hPPARδΔAF2, PPARα-KO and PPARδ-KO) it was be possible to show how the genetic ablation of the two

of the PPAR subtypes or over-expression of human PPARδ or dominant negative affect the transcriptional response of PPAR target genes in a genome wide manner.

7.2 Results

9-11-week old male and female C57BL/6 background mice (5 animals/group) were placed on different diets. The groups are presented in table 7.1.

Table 7.1 Genetic and treatment groups for microarray experiment.

	Genotype					
Diets groups	non-transgenic	non-transgenic	hPPARδ	hPPARδΔAF2	PPARα-KO	PPARδ-KO
Control	RMI	RMI + 0.25% I3C	RMI + 0.25% I3C	RMI + 0.25% I3C	RMI	RMI
Treatment	RMI + 0.0025 GW501516	RMI + 0.25% I3C + 0.0025 GW501516	RMI + 0.25% I3C + 0.0025 GW501516	RMI + 0.25% I3C + 0.0025 GW501516	RMI + 0.0025 GW501516	RMI + 0.0025 GW501516

After 5 days of treatment, mice were sacrificed, livers harvested and gene expression from this organ analyzed utilizing microarrays. Out of 30854 genes (consisting of 45281 probes) available on the microarray chips, 9548 reached intensities higher from background with a mean P value over the 60 samples of <0.1. This broad selection was required to not exclude genes that were non-expressed in specific samples eg. the knockouts, or where expression was only significantly detectable in the induced samples.

Several distinct patterns of transcriptional cooperation between PPARα and PPARδ were found when analyzing microarray data. The first pattern of transcriptional regulation was revealed where hPPARδ or PPARα-KO

genotypes mediate marked change of expression of 119 genes out of 9548 upon GW501516 treatment (P value <0.01, FDR <0.05), from which 61 were significantly up-regulated and 58 down-regulated. In this group, the change of proportion in the abundance of these two PPARs was in favour of PPARδ, through over-expression of hPPARδ or genetic KO of PPARα relieves or strengthens the transcriptional repression, Therefore for these genes PPARα is working as a gatekeeper of PPARδ target genes. Typical examples of these genes and one of the top hits are: cyclin-dependent kinase inhibitor 1A (Cdkn1a) regulating cyclin-dependent protein kinase involved in cell cycle regulation (Fig 7.1 A), G0/G1 switch gene 2 (G0s2), which plays role in positive regulation of apoptotic process (Fig7.1 B) or regulator of G-protein signalling 16 (Rgs16) (Fig 7.1 C).

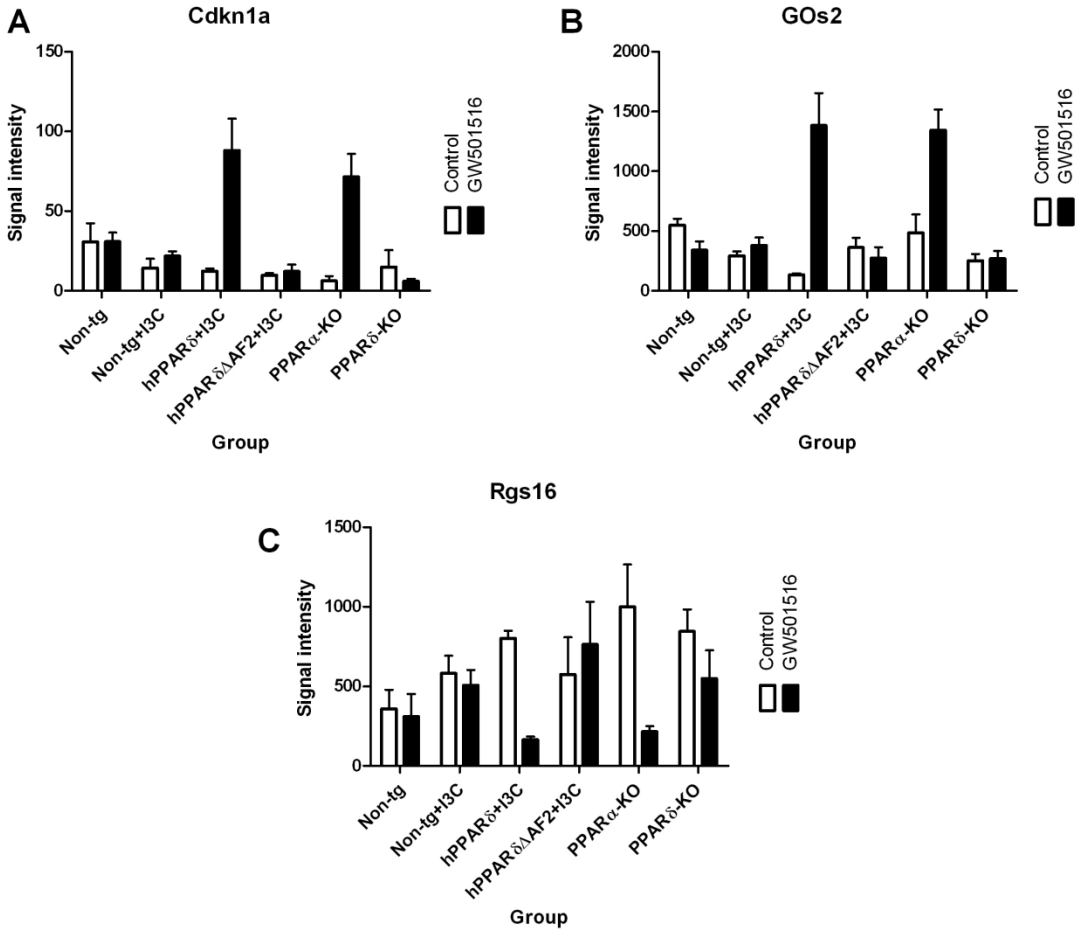

Figure 7.1 Three selected genes presenting how hepatic change of proportion in PPARs in favour of PPARδ, influences the expression of the target genes upon GW501516 treatment. n=5 mice/group. Data are expressed as means ± SEM.

A second mode of regulation was found when sets of genes were identified where their expression was significantly dependent on GW501516 treatment but not influenced by PPARα ablation. From 25 genes altogether only, 18 were up-regulated and 6 down-regulated by GW501516 treatment, including PPARα-KO genotype mice (P value <0.01, FDR <0.05). The genes which were up-regulated were usually more pronounced in hPPARδ mice, but normally also reactive to GW501516 in wild-type mice. In this group, knocking out PPARδ and prevented GW501516 from invoking transcriptional response. 3

examples of up-regulated genes include: carnitine acetyltransferase (Crat), a mitochondrial protein involved in fatty acid beta-oxidation (Fig 7.2 A), Angiopoietin-related protein 4 (Angptl4), secretory protein, well known PPARδ target gene, which plays essential role in triglyceride homeostasis (Fig. 7.2 B) and ST3 beta-galactoside alpha-2,3-sialyltransferase 5 (St3gal5) engaged in carbohydrate metabolic process (Fig. 7.2 C). This group could be described as PPARδ dependent, but PPARα independent genes.

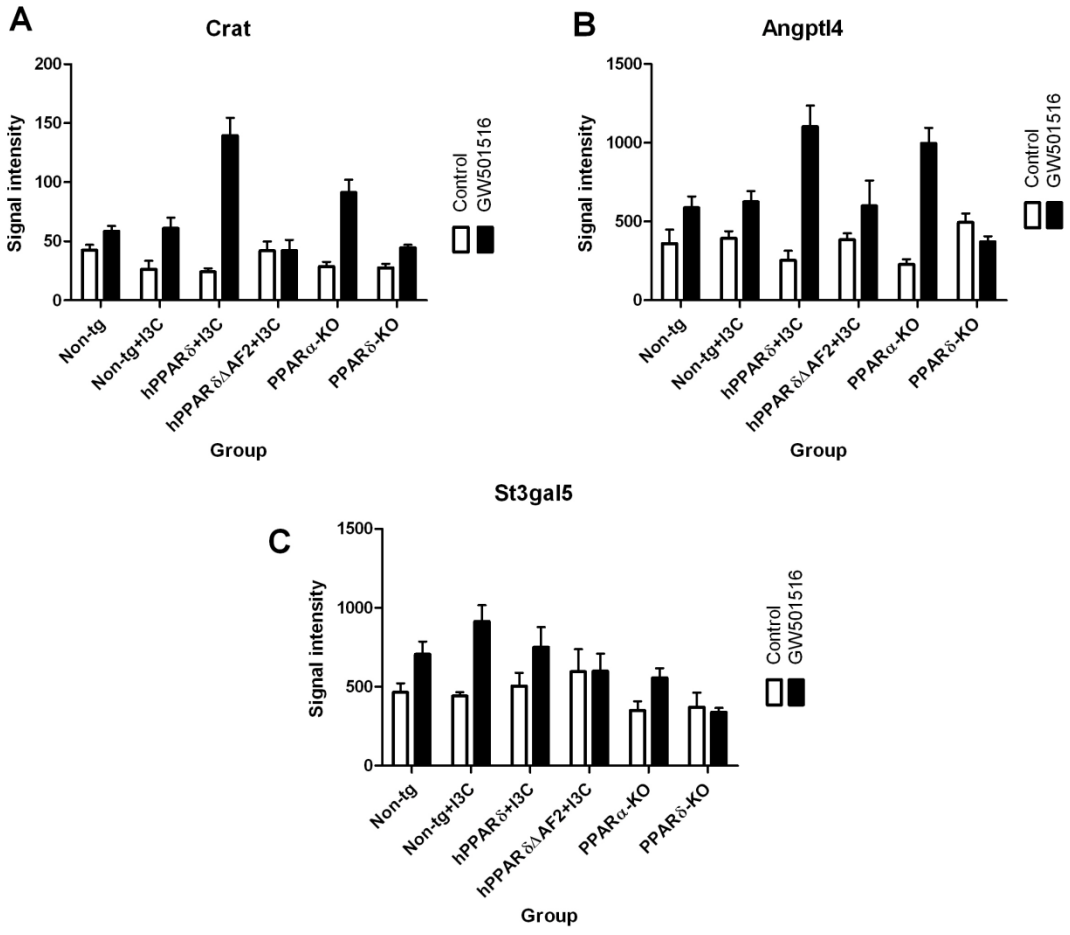

Figure 7.2 Examples of the genes reactive to GW501516 treatment, but not affected by PPARα knock out. The hPPARδΔAF2 generally blocked the GW501516 response in this class of genes. n=5 mice/group. Data are expressed as means ± SEM

The third pattern of transcriptional regulation emerged when ablation of PPARα caused down-regulation of the basal levels of genes, which are reactive to the PPARδ agonist. In this case, removal of PPARα from the “system”, works stronger than simple antagonism, where typical antagonist generally only prevents the activation of the transcription factor preventing the up or down regulation of the target genes, leaving the basal levels of given transcriptome unchanged. Here, PPARα genetic ablation, causes even reduction of the basal levels of the target mRNA. 530 genes were identified (P value <0.01, FDR <0.05), which have reached more than 0.75 fold reduction in the basal level expression in PPARα-KO mice, when compared to wild-type or humanized mice. One of the top 3 genes with greatest reduction of its mRNA levels when PPARα was knocked out were: galactose-3-O-sulfotransferase 1 (Gal3st1), involved in glycolipid biosynthetic processes (Fig. 7.3 A), serine incorporator 2 (Serinc2), a L-serine transmembrane transporter (Fig. 7.3 B) and AMP-activated protein kinase, gamma 2 (Prkag2), which plays role in regulation of fatty acid metabolic processes (Fig. 7.3 C).

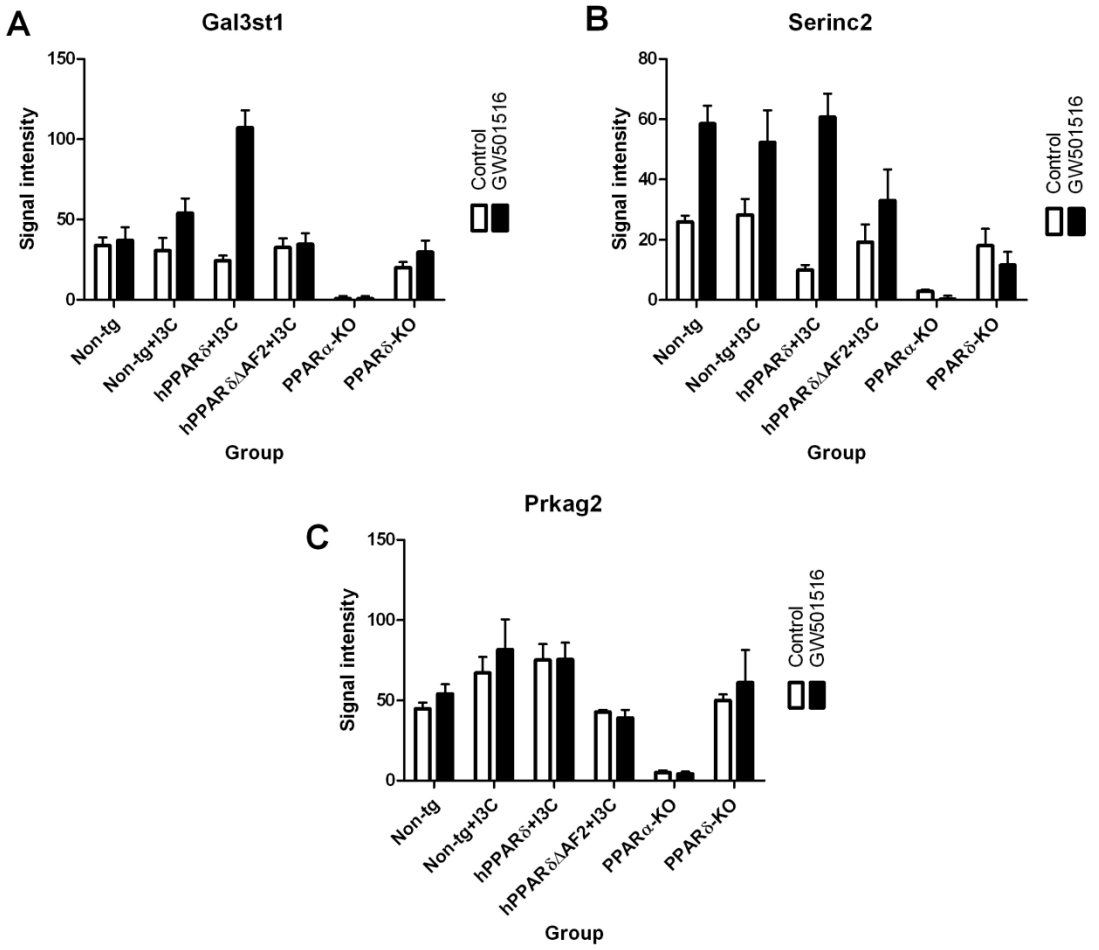

Fig 7.3. PPARα genetic knock out down-regulates the basal levels of the defined sets of genes. n=5 mice/group. Data are expressed as means ± SEM

The fourth pattern found is paralleled with the previous one, but in this case the PPARδ knockout affects the basal levels of defined sets of genes. 1564 genes were found to be significantly affected by down-regulation caused by genetic removal of PPARδ (P value <0.01, FDR <0.05, more than 0.75 fold reduction in the basal level expression). Typical examples include: zinc finger and BTB domain containing 7a (Zbtb7a), which plays role in regulation of transcription (Fig. 7.4 A), essential meiotic endonuclease 1 homolog 2 (Eme2) with its role in DNA recombination and repair (Fig. 7.4 B) and T-cell lymphoma invasion and metastasis 2 (Tiam2) involved in apoptotic processes (Fig. 7.4 C).

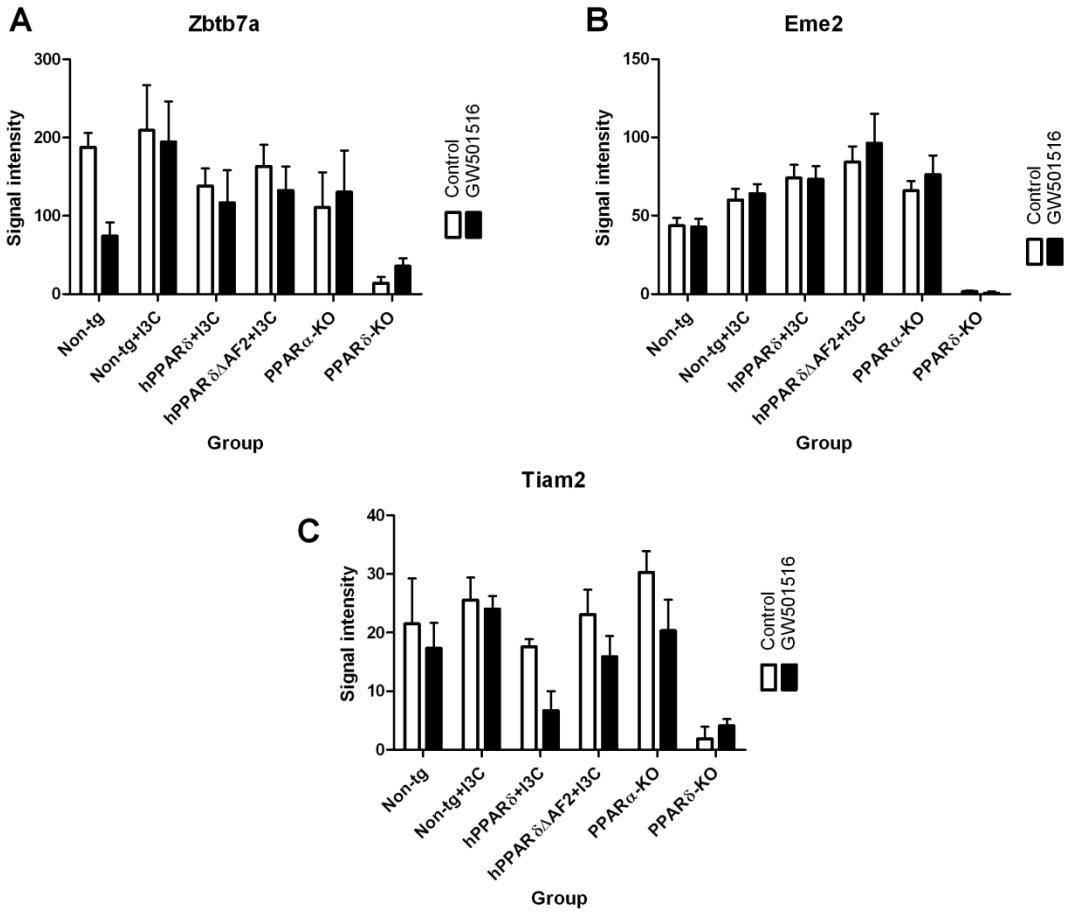

Figure 7.4 PPARδ genetic knock out down-regulates the basal levels of the defined sets of genes. n=5 mice/group. Data are expressed as means ± SEM

In summary in this group, PPARδ functional protein is working as constitutive transcription factor for a relatively large group of genes, but interestingly, these genes are not generally reactive to GW501516.

Another pattern, the fifth, was revealed when analysis have shown that both PPARα and PPARδ are essential for defined set of genes expression. 853 were identified (P value <0.01, FDR <0.05, more than 0.75 fold reduction in the basal level expression). Most of these genes were not reactive to GW501516 treatment, but their levels were highly dependent on simultaneous presence of

both receptors. Typical examples of this transcriptional collaboration include: glutathione S-transferase, alpha 2 (Gsta2) encoding xenobiotic metabolizing protein (Fig. 7.5 A), phosphatidic acid phosphatase type 2C (Ppap2c), which its product is involved in sphingolipid biosynthesis (Fig. 7.5 B) and heat shock protein 8 (Hspb8), induced as a response to stress (Fig. 7.5 C). These genes, although in the examples shown not reactive to GW501516 require functional proteins of both receptors for stable, basal levels of their transcription. Interestingly, only part of this gene group overlaps with two previous groups were PPARα or PPARδ were needed for basal expression.

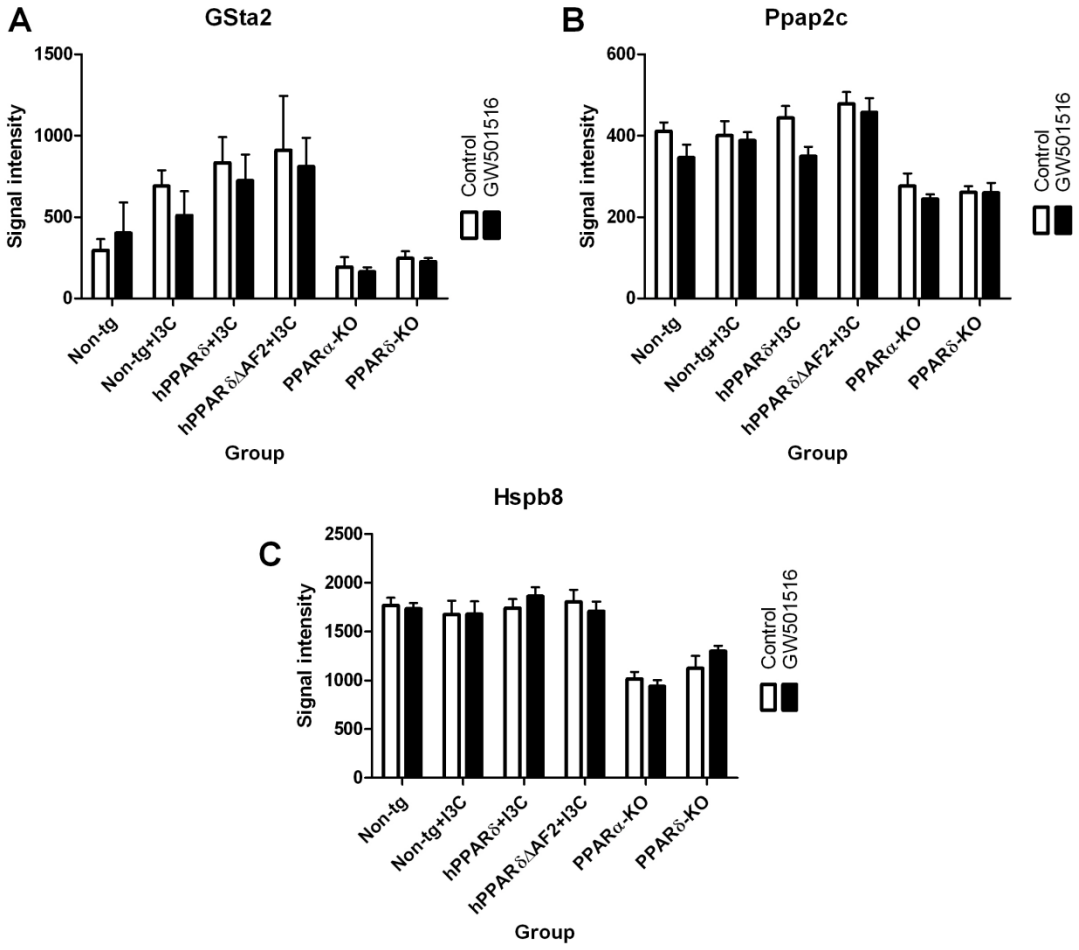

Figure 7.5 Both PPARα and PPARδ are needed for target gene expression. Ablation of both receptors causes down-regulation of transcription. n=5 mice/group. Data are expressed as means ± SEM

The last, sixth group identified with the distinctive pattern of expression indicate that both PPARα and PPARδ are required in the normal condition for repression of the defined set of gene. Elimination of these both receptors from the hepatic environment, causes marked up-regulation of 275 genes (minimum 1,5 fold up-regulation, P value <0.01, FDR <0.05). The three examples of this pattern are: abhydrolase domain containing 1 (Abhd1), possessing carboxylesterase activity (Fig. 7.6), ATPase, H+ transporting, lysosomal V1 subunit G1 (Atp6v1g1) with its product involved in transmembrane transport (Fig. 7.6 b) and acetyl-Coenzyme A acetyltransferase 3 (Acat3), with its protein working as a part of cellular response to fatty acid (Fig. 7.6 C). In this group of genes in normal condition, PPARα and PPARδ work as powerful repressors.

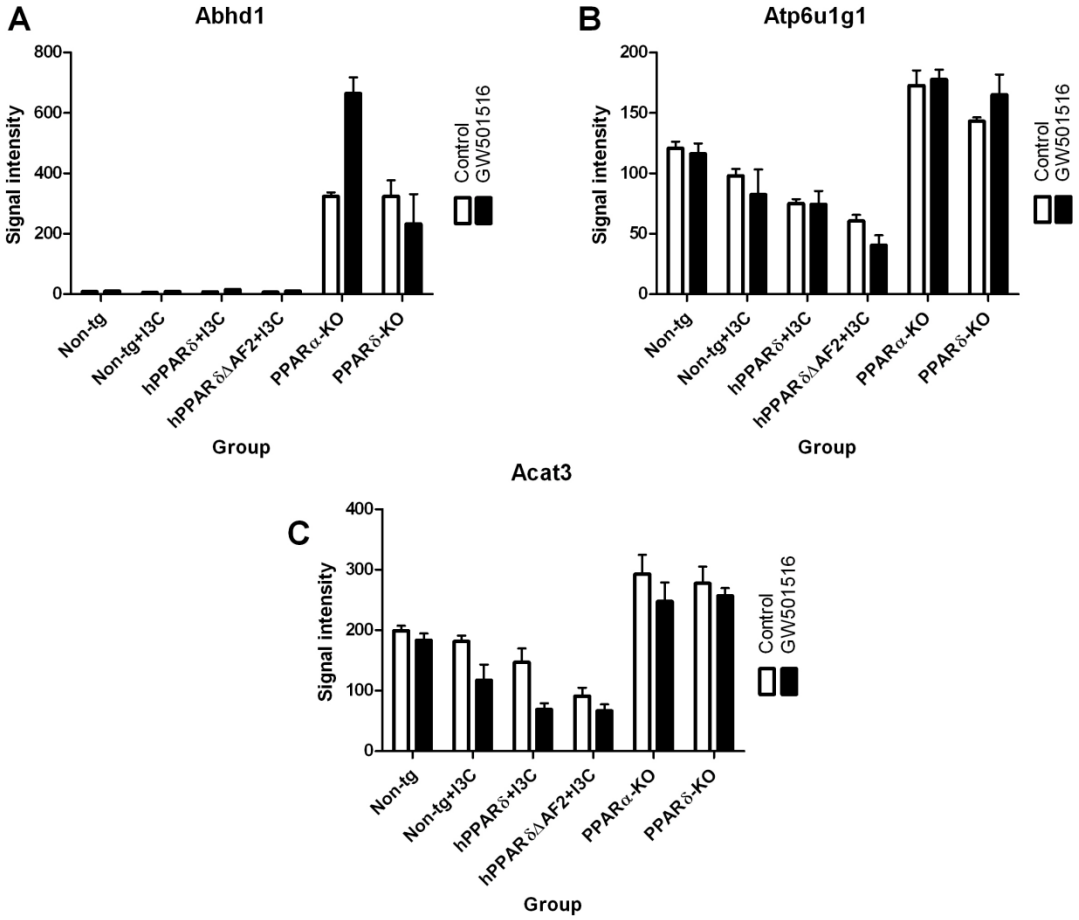

Figure 7.6 PPARα and PPARδ both needed for repression of the genes, which are generally non-responsive to GW501516 treatment. n=5 mice/group. Data are expressed as means ± SEM

Summary of PPARα and PPARδ coordination in regulation of gene expression is shown in table 7.2

Table 7.2 Modes of PPARα and PPARδ cooperation in regulation of gene expression in the liver.

Mode	Description	Figure
PPARα as a repressor for PPARδ target genes	hPPARδ over-expression or PPARα-knockout mediate marked change of expression of defined set of genes	7.1

PPARδ-only specific gene targets	Gene expression dependent on GW501516 treatment but not influenced by PPARα ablation	7.2
PPARα as a "co-activator" for PPARδ target genes	Ablation of PPARα caused down-regulation of basal levels of the genes, which are reactive to PPARδ agonist	7.3
PPARδ repressive action needed for stable expression	PPARδ knockout down-regulates basal levels of defined sets of genes	7.4
PPARα and PPARδ as cooperative "promoters" of gene expression	Both PPARα and PPARδ are essential for defined set of genes expression	7.5
Both PPARα and PPARδ as copperative constitutive repressors	Both PPARα and PPARδ are in normal condition required for repression of the defined set of gene	7.6

Apart from using microarray data for deciphering transcriptional relationship and collaboration between PPARα and PPARδ in regulation of gene expression, the hepatic expression profile was also used to predict prospective weight loss or hepatic steatosis in mice as a response to GW501516 treatment. Group means for weight gain (% of initial body mass) and hepatic triglyceride levels at point of 2 weeks were formed from data from earlier experiments described in previous chapters. They reflected exactly the 6 groups shown in this chapter. In details: non-transgenic mice fed control or diet supplemented in GW501516, non-transgenic, hPPARδ and hPPARδΔAF2 fed control + 0.25% I3C or diet supplemented with 0.25% I3C + 0.0025% GW501516; PPARα-KO and PPARδ-KO fed control or diet supplemented in GW501516 (w/w). Phenotypic group means from the experimental 2 weeks time point were then correlated with hepatic gene expression data obtained from 5 days microarray experiment.

29 genes were identified, whose pattern of expression was significantly correlated with rate of weight gain established from previous independent experiments, however, no genes were found (FDR below 0.05), which would be significantly correlated with level of hepatic steatosis. Several genes involved in transmembrane transport were identified such as Abcc3 (Fig. 7.7 A), which plays role in bile acid and bile salt transport and has been reported in diabetic phenotype, Slc19a1 (Fig. 7.7 B) encoding folate transporter and Slc25a10 (Fig. 7.7 C), the mitochondrial malate and succinate carrier. Other examples involve genes involved in lymphocyte differentiation like Ly6d (Fig. 7.7 D), which has been previously associated with the degree of hepatic steatosis in mice. Member of perilipin family S3-12, (Fig. 7.7 E), involved in coating intracellular lipid droplets and an adipogenic marker, gene expression governing genes such as Taf1d or cell growth factors like Igf1 (Fig. 7.7 F). All, but Igf1 turned out to be negatively correlated with weight gain. Full list of the genes is presented in Table 7.3. Examples of pattern of expression and correlation graphs of chosen genes are shown in Figure 7.7

Table 7.3 List of the genes involved in promoting or assisting weight loss altered by GW501516 treatment in mouse liver and identified through correlation tests between microarray hepatic expression data from 5 days vs weight gain rate (% of initial body mass) after 2 weeks from several independent experiments.

Gene symbol	Enterez gene ID	Pearson R	P value	False discovery rate	Gene description	Biological process
Abcc3	76408	-0.9649	3.97E-07	0.003	ATP-binding cassette, sub-family C (CFTR/MRP), member 3	transmembrane transport
Slc19a1	20509	-0.9355	7.91E-06	0.021	Solute carrier family 19 (sodium/hydrogen exchanger), member 1	transmembrane transport

Slc25a10	27376	-0.9211	2.10E-05	0.034	Solute carrier family 25 (mitochondrial carrier, dicarboxylate transporter), member 10	transmembrane transport
Abcc4	239273	-0.9096	4.07E-05	0.033	ATP-binding cassette, sub-family C (CFTR/MRP), member 4	transmembrane transport
Slc16a5	217316	-0.9003	6.53E-05	0.035	Solute carrier family 16 (monocarboxylic acid transporters), member 5	transmembrane transport
Srd5a3	57357	-0.9229	1.88E-05	0.038	Steroid 5 alpha-reductase 3	steroid catabolism
Cbr1	12408	-0.9055	5.04E-05	0.034	Carbonyl reductase 1	redox recations
Grpel1	17713	-0.8953	8.31E-05	0.035	GrpE-like 1, nuclear gene encoding mitochondrial protein	protein anabolism
Ripk4	72388	-0.9023	5.94E-05	0.034	Receptor-interacting serine-threonine kinase 4	phosphorylation
Serhl	68607	-0.9201	2.24E-05	0.03	Serine hydrolase-like (Serhl), mRNA.	peroxisome function
Atxn10	54138	-0.8938	8.87E-05	0.034	Ataxin 10	nervous system development
Chchd6	66098	-0.8978	7.38E-05	0.033	Coiled-coil-helix-coiled-coil-helix domain containing 6	mitochondrial function
Ly6d	17068	-0.9099	4.02E-05	0.036	Lymphocyte antigen 6 complex, locus D	lymphocyte differentiation
Unc119	22248	-0.9030	5.75E-05	0.035	Unc-119 homolog (C. elegans)	lymphocyte differentiation
S3-12	57435	-0.8770	0.00018	0.048	Plasma membrane associated protein, S3-12	lipid droplets coating
Gns	75612	-0.8952	8.33E-05	0.033	Glucosamine (N-acetyl)-6-sulfatase	glycosaminoglycan metabolic process
Gal3st1	53897	-0.8796	0.000163	0.05	Galactose-3-O-sulfotransferase 1	glycolipid synthesis
Cpsf1	94230	-0.8935	8.99E-05	0.033	Cleavage and polyadenylation specific factor 1	gene expression
Taf1d	75316	-0.8913	9.93E-05	0.033	TATA box binding protein (Tbp)-associated factor, RNA polymerase I, D (Taf1d), transcript variant 2, mRNA.	gene expression
Sox12	20667	-0.8786	0.000169	0.05	SRY-box containing gene 12	gene expression
S100a13	20196	-0.9524	1.78E-06	0.007	S100 calcium binding protein A13	cytokine secretion
Igf1	16000	0.8777	0.000175	0.048	Insulin-like growth factor 1	cell growth
Nrg4	83961	-0.8991	6.95E-05	0.033	Neuregulin 4	Cell growth
Pmm1	29858	-0.9147	3.08E-05	0.035	Phosphomannomutase 1P	carbohydrate metabolism
Prune	229589	-0.8866	0.000122	0.039	Prune homolog (Drosophila)	carbohydrate metabolism
Tmem120a	215210	-0.9082	4.40E-05	0.032	Transmembrane protein 120A	
C230029F24Rik	442837	-0.9141	3.19E-05	0.032	PREDICTED: Mus musculus RIKEN cDNA C230029F24 gene	
1600032L17Rik		-0.9001	6.62E-05	0.033	PREDICTED: Mus musculus RIKEN cDNA 1600032L17 gene	
2410012H22Rik	69747	-0.8784	0.00017	0.049	PREDICTED: Mus musculus RIKEN cDNA 2410012H22 gene	

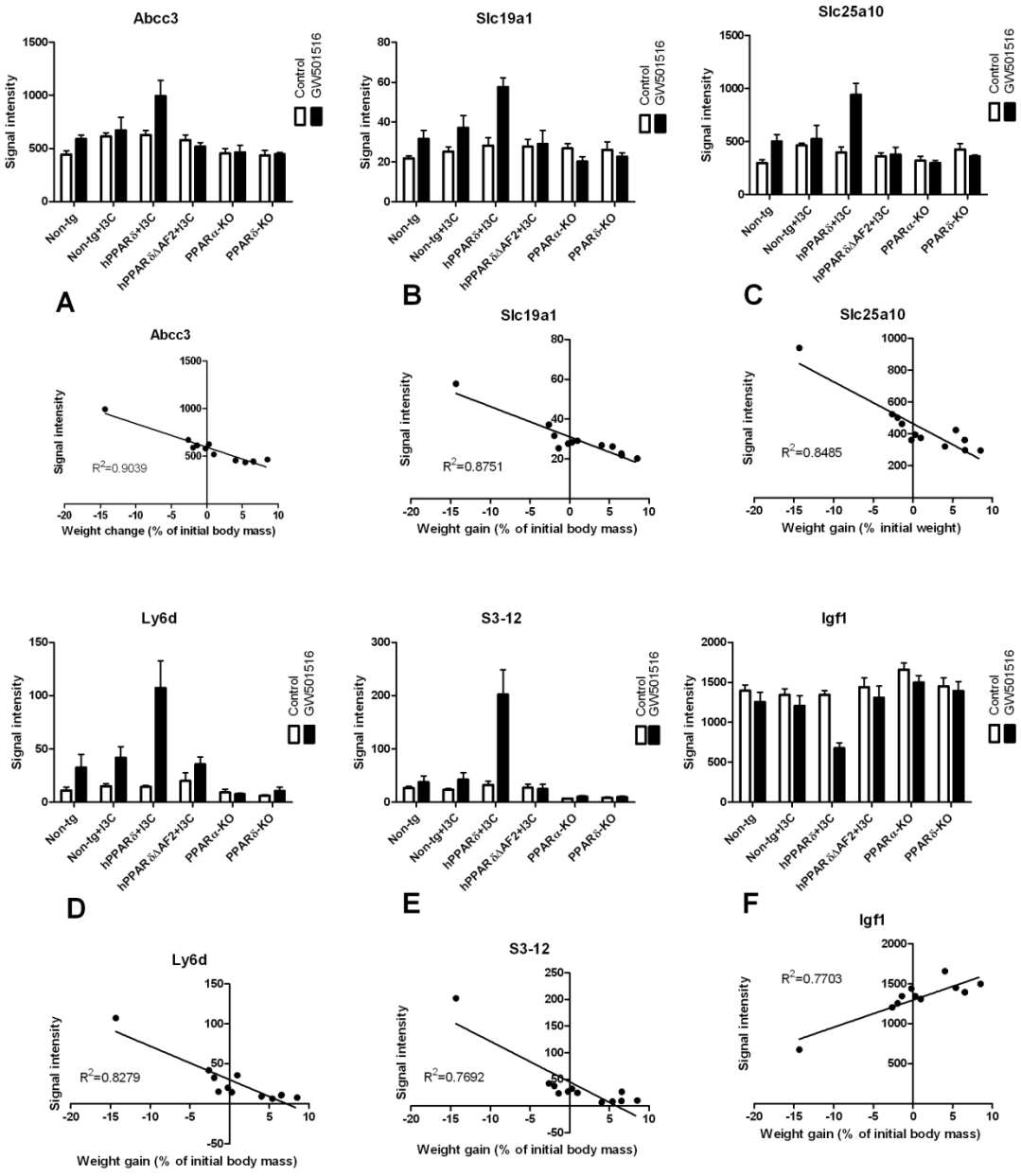

Figure 7.7 Genome-wide transcriptional profiling of GW501516 effects in various genetic models reveals strong correlation between liver gene expression after 5 days and successive weight gain rate after 2 weeks. Pattern of hepatic gene expression across the experimental genotypes in 5 days (bars graphs) and 2 weeks weight gain vs 5-days expression data for Abcc3 (A), Slc19a1 (B), Slc25a10 (C), Ly6d (D), S3-12 (E), Igf1(F) (scatter graphs). Each point on the scatter graph represents mean value of each group (control or treated vs expression value) for given gene. n=5 mice/group. Data are expressed as means ± SEM

7.3 Discussion

In vivo model for investigation of PPARα and PPARδ cooperation in transcriptional regulation of gene expression.

In nuclear receptor biology, initial activity of these transcription factors, translate subsequently into metabolic and physiological changes. Although the time course of the experiment was 5 days only, too short to induce major physiological alterations seen in the longer studies in previous chapters, it turned out to be sufficient enough to expose important gene expression mechanisms. The other advantage of 5 days study only was also preservation of nuclear-receptor-based transcriptional activity of whole sets of genes, not influenced yet by various physiological feedback loops and homeostatic mechanisms, which are likely to occur in later stages of chronic ligand treatment. One of the aims of this chapter was to show elaborate cooperation between PPARα and PPARδ in transcriptional regulation of their target genes. Indeed, several distinctive ways have emerged how these two receptors in close collaboration regulate mRNA synthesis of defined set of genes. One of the most intriguing was when over-expression of hPPARδ or deletion of PPARα had a similar and profound effect on mostly up-regulation of a certain set of genes. In both cases PPARδ signalling was becoming dominant over PPARα activity. Clearly it is not only the activation status of these receptors that determines the transcriptional outcome of target genes, but also relative proportion of the subtypes that is present in the system. In contrast to the mouse, human liver is known to have PPARδ dominating over PPARα quantitatively [93,125]. As the figure 7.1 shows, for given sets of genes, over-expression of hPPARδ might indeed “humanize” rodent liver, at least in relation

to PPARs abundance proportion. Although in the liver of non-transgenic mice the level of expression of PPARα is higher than PPARδ expression [12], during fasting the situation changes quantitatively even more in favour of PPARα [118]. Interestingly Escher et al showed that this physiological/nutritional change of proportion is due to the PPARδ, which is down-regulated in the liver in prolonged fasting. After an overnight fast, the authors observed a similar decrease in hepatic PPARδ mRNA expression in wild-type and PPARα null-mutant mice by up to 80% [173]. Thus, they concluded that nutritional regulation of hepatic PPARδ mRNA expression by fasting is independent of the activity of PPARα. Here we show that although the level of expression of PPARδ mRNA might be might be not dependent on PPARα presence or activity in the liver, the target genes for PPARδ are highly influenced by absence of PPARα. One might speculate that physiological decreases in the levels of PPARδ in the rodent and possibly in human liver during fasting might be a metabolic adaptation for “sensitizing PPARα” and subsequently facilitating its transcriptional activity along with ligand availability and abundance of its nuclear-receptor protein. It is equally possible that an analogous mechanism exists for PPARδ being “sensitized” by PPARα absence (KO animals) or great reduction in proportions (hPPARδ over-expression), that he have just shown using DNA microarrays. However, such hypothesis would assume that at least in certain range of genes and molecular events, these two receptors have antagonistic properties. The work done by Gustafsson et al seems to confirm this, by showing that genetic ablation of PPARδ removes its role in retarding of PPARα and PPARγ signalling [148].

Summarizing, the expression patterns shown in groups 1 and 2 (Fig 7.1 and Fig 7.2 respectively), are quite similar and partly overlap, with the main difference that the first set of genes is not reactive to GW501516 in non-transgenic animals, unlike the latter group, where their up or down-regulation is clearly noticeable in wild-type animals as well.

Expression patterns 3 and 4 (Fig 7.3 and Fig 7.4 respectively), where PPARα or PPARδ genetic ablation causes marked down-regulation of mRNA of specific groups of genes is fully consistent with the findings described in work performed by Adhikary et al [153]. The authors clearly find that siRNA silencing of PPARδ down-regulates given sets of genes, but these genes are normally not reactive to PPARδ ligand. It is tempting to speculate whether therapeutic implementation of such mechanism of transcriptional control would not provide stronger effects than simple use of specific antagonist, which only prevents the receptor from activation, stabilizing its basal transcriptional activity [174].

Even more pronounced effect of transcriptional regulation highly dependent on close PPARα and PPARδ cooperation emerges from expression patterns 5 and 6 (where basal level of specific groups of the genes was down-regulated or up-regulated, respectively) after simultaneous genetic ablation of both receptors. Already cited in the introduction for this chapter recent report from Bugge et al shows similar phenomenon. In short: genetic KO of nuclear receptors Rev-erbα together with closely related Rev-erbβ in mouse embryonic fibroblasts, renders circadian clock proteins Bmal1 and Cry1 expression arrhythmic. It was in contrast to the mild effect on these clock genes, when only single deletion of Rev-erbα or Rev-erbβ was present. The authors conclude that this subtype collaboration is unusual among nuclear receptors but common

among core clock proteins [172]. In our work we show that this cooperation extends also to peroxisome proliferator-activated receptors. It is also characteristic that genetic ablation of PPARα or PPARδ causes always two-way transcriptional reaction: up-regulation and down-regulation of given sets of genes. It is certainly individual case for each gene what is exact mechanism how this is achieved. This subject was partly discussed in the previous chapter, but without doubt it consists of complex interaction between various co-repressors and co-activators for which nuclear receptor in normal condition is either a constitutive direct repressor or repressor of repressors. Summarizing, the picture which emerges from our data shows that abundance of nuclear receptor in "unliganded", dormant state is equally important for gene expression as ligand binding and its subsequent downstream signalling. However, the subsets of the genes controlled and modulated by dormant nuclear receptor do not overlap fully with groups controlled by agonist activity.

Early hepatic gene expression predicts rate of subsequent weight loss upon GW501516 treatment.

Genome-wide transcriptional profiling has also demonstrated that early transcriptional actions in the liver involving PPARα-PPARδ tandem activities, translates directly into phenotypic events in later stages. By comparing transcriptional profile of various strains of mice fed GW501516 for 5 days we were able to correlate it directly with weight loss or gain phenotype from two-weeks. However, significant correlation failed to occur with the hepatic triglyceride profiles defined for mice from previous experiments. It confirms the already mentioned hypothesis that reduction in peripheral fat deposits and

therefore consequent weight loss precedes and is essential for hepatic lipid accumulation observed in our mice genetic models upon GW501516 treatment.

The most numerous group of the genes significantly correlated with the weight loss turned out to be transmembrane transporters. Abcc3 was one of the most significantly associated genes. In study done by Hardwick et al hepatic mRNA for Abcc3 was found to be elevated in human liver samples with confirmed non-alcoholic liver fatty live disease (NAFLD). However in the same study immunohistochemical staining of Abcc3 revealed no alterations in cellular localization during NAFLD progression [175]. Additionally, More et al found that mRNA for Abcc3 and Abcc4 transporters in livers of obese *db/db* mice (genetic deletion of leptin receptor) were expressed more than 2 fold higher, than in C57BKS wild-type mice [176]. Abcc3 transporter along with Slc19a1, which was also highly associated with forthcoming weight loss in our data, is also a significant drug transporter, an important factor in response to methotrexate, a drug used for treatment of juvenile idiopathic arthritis [177]. Another member from our transporter hits: Slc25a10 was found to be facilitating *de novo* fatty acid synthesis in white adipose tissue [178]. Slc25a10 supplies malate for citrate transport. Citrate is required for forming acetyl-CaA a key component of lipogenesis [178]. Although in our model of hepatic triglyceride accumulation the expression of fatty acid synthase plays a minimal role, and FAS is considered the rate limiting step in *de novo* lipogenesis in the liver, it remains a possibility that some aspects of lipogeneis are raised in our model; However, this transmembrane transporter is also involved in glucose metabolism. Slc25a10 was shown to be essential for glucose stimulated insulin secretion (GSIS). In work published by Huypens et al, an adenovirus expressing a siRNA against

Slc25a10 inhibited GSIS [179]. S3-12 was also identified as being highly correlated with weight loss. S3-12 is a member of perilipin family with function similar to ADRP. This is very interesting as we demonstrated that, whereas ADRP expression was highly correlated with the level of hepatic lipids in non-transgenic, hPPARδ and hPPARδΔAF2 mice, it was still highly inducible in PPARα-KO livers where lipid accumulation was absent (Fig 6.5A and Fig 6.6A). On the other hand, S3-12 expression was tempered efficiently by genetic KO of both receptors (Fig. 7E). S3-12 was previously shown to be involved in fatty liver phenotype when PPARγ1 was over-expressed in transgenic mouse [180], is induced by fasting in PPARα null mice [12], and in our study, we also demonstrate it as a PPARδ target gene in the mouse liver.

Another strong candidate for fatty liver phenotype, although not directly associated with triglyceride accumulation is Ly6d a part of lymphocyte antigen complex. In microarray analysis of liver transcriptome from apoE-deficient mice fed a western-type diet enriched with linoleic acid isomers (known to promote fatty liver in mice), the Ly6d was found among other 9 genes highly associated with the degree of steatosis [181]. The mentioned study's duration was 12 weeks and our experiment was performed for 5 days only. It shows how consistent the elevated expression of Ly6d is in molecular events leading to liver triglyceride accumulation. If future studies confirm up-regulation of Ly6d in the early stages of hepatic steatosis, it might serve as a solid biomarker for fatty liver.

The only significantly negative correlation of expression of a gene with subsequent weight loss was Igf1. Low levels of insulin growth factor 1 were found in sera of patients with hepatic steatosis and the authors concluded that

this association was independent of alcohol consumption [182]. Also Estep et al reported that miRNA specimens associated among others to IGF1, were differentially expressed in white adipose tissue between NASH and non-NASH patients. [183].

In summary, utilizing microarray technique we were able to identify a group of hepatic genes in which expression was strongly correlated with future metabolic events leading to GW501516-induced reduction in body weight and consequent triglyceride accumulation in the mouse liver. Additionally, close transcriptional mutual collaboration and antagonistic properties were revealed between PPARα and PPARδ in the control of gene expression in the liver.

Chapter 8 Conclusions

The work shown in this thesis has focused on deciphering the role of peroxisome proliferator activated receptor delta in the control of body weight and hepatic lipid homeostasis. The results of this analysis will contribute to the overall assessment of the drug safety profile of a new generation of drugs that selectively target PPARδ.

Up to date, there are still no drugs available on the market, which selectively target PPARδ receptor. However, synthetic agonists for PPARδ were shown to attenuate multiple metabolic abnormalities normally associated with the metabolic syndrome in humans, by lowering fasting and postprandial plasma triglyceride, LDL cholesterol, FFA and increase HDL cholesterol [116,184]. At the molecular level PPARδ activation has been shown to modulate the expression of a number of genes in muscle, which are involved in lipid uptake, storage and metabolism and also in glucose homeostasis [22,113,123,124,185]. Apart from its important role in skeletal muscle metabolism, evidence is accumulating that PPARδ, plays equally vital role in the management of lipid turnover also in the liver [129,130,186,187].

At the beginning of this work there were contradicting reports on PPARδ agonism promoting or protecting from fatty liver phenotype. Giving the promising role of PPARδ agonists in improving of some aspects of metabolic syndrome and susceptibility of individuals with this syndrome or type 2 diabetes to fatty liver steatosis, this thesis along with the newest published reports will add to understanding of the less characterized, but essential from toxicological perspective role of selective PPARδ synthetic agonists in liver lipid response.

Effect of PPARδ agonism on body weight

PPARδ synthetic agonists retard the rate of weight gain in rodent models of high-fat diet–induced obesity [81,123,188] and the experimental approach presented in this thesis focused on *in vivo* pharmacological studies using various genetic models of mice including humanized animals.

However, unlike in the rodent model, 4-month treatment of obese rhesus monkeys with different doses of GW501516 did not affect body weight [127]. Therefore it is still an open question, if long-term delivery of drugs based on PPARδ ligands will control body weight in primates. For example in a recent study conducted in men, in 12 weeks of administration of GW501516, loss in body weight was not apparent. Although an increase in body reduction was seen for the highest dose group (10 mg), the data collected did not allow characterization of the weight change composition, which potentially includes fat, muscle mass, glycogen storage, or water [184]. The importance of body composition change upon GW501516 action was revealed when examining body fat and lean mass in transgenic and non-transgenic mice shown in chapter 4. The greatest input to total weight reduction in hPPARδ mice was white fat mass decrease, which is consistent with findings from Wang et al, where WAT and BAT-specific expression of constitutively active VP16-PPARδ protein caused almost complete reduction in adipose tissue mass [81]. Although PPARδ is expressed ubiquitously in rodents and in humans, its expression in skeletal muscle is 10 and 50-fold higher levels compared with PPARα and PPARγ, respectively [189]. Also, muscle accounts for approximately 80% of insulin-stimulated glucose uptake, therefore it might be assumed that the main energy requirement for lipids, when glucose is low, is coming from the muscle.

And therefore the signalling from muscle partly stimulated by PPARδ transcriptional activity in this organ will be the main cause to drive adipose tissue lipolysis. However, I3C-induced over-expression of hPPARδ in our transgenic mice was almost exclusively restricted to liver (Fig. 4.9) and partly to other organs [140], but in the muscle, transgene expression was extremely low when compared to mentioned organs. Additionally, the endogenous mouse PPARδ was still active in muscle of transgenic mice, but the difference in weight loss between hPPARδ and wild-type animals was still very significant (Fig. 4.1A, 4.2 A and B). And the lack of body mass reduction in hPPARδΔAF2 animals treated with GW501516, which also had expression of the transgene mostly confined to liver, seems to confirm importance of liver. Therefore, liver emerges as a key organ in PPARδ-stimulated reduction in weight gain. The hypothesis suggested by Perreault et al [144] that PPAR agonists might suppress the appetite and therefore this is one of the primary reasons for weigh gain reduction was partly confirmed in wild-type animals, but not supported in experimental evidence from hPPARδ animals in this thesis.

In most cases, the WAT lipolysis occurs upon hormonal stimulation [15]. But the pharmacological stimulation of weight reduction by use of PPARδ synthetic agonists does not necessarily absorb hormonal-induced initiation of triglyceride hydrolysis and release. A proposed candidate factor linking hepatic PPARδ activity and the nutrient and hormone dependent and independent WAT lipolysis, is ANGPTL4. A wealth of evidence from *in vivo* studies shows that ANGPTL4, a glycoprotein that is secreted by the WAT and liver, plays a fundamental and essential role in not only preventing the uptake of triglyceride rich lipoproteins by WAT, but also in adipocyte lipolysis [149,166]. As it was

shown by Grey et al ANGPTL4 positively modulates the activity of cAMP-dependent protein kinase A (PKA). And the phosphorylation by this kinase is essential for activation of triglyceride hydrolytic enzymes [166]. Recently, ANGPTL4 supportive role in mediating triglyceride hydrolysis and release of FFA from adipocytes was linked with PPARδ activity [12,157,165], which is not surprising as ANGPTL4 is PPARδ target gene. By using RT-PCR we demonstrated PPARδ-mediated up-regulation of ANGPTL4 in livers of wild-type animals after 2 weeks or PPARα-KO mice at every measured time point (Fig. 4.11E and Fig. 6.6 C respectively), but this change was absent in mice expressing hPPARδ after 2 weeks (Fig. 4.11E). However, microarray data evidently show that hPPARδ animals fed GW501516 along with the wild-type animals and PPARα-KO, but not the hPPARδΔAF2 and PPARδ-KO mice had significantly up-regulated levels of ANGPTL4 (Fig. 7.2 B). These findings also indicate that ANGPTL4 expression is not dependent on PPARα signalling, but as it was shown using PPARα-KO animals, PPARα presence is required for GW501516-stimulated lipolysis nevertheless. Therefore PPARδ regulation of ANGPTL4 is not sufficient to account for GW501516 mediated lipolysis.

Together these findings suggest that PPARδ activation can not only induce efficient fat burning [31], but also seems to initiate white adipose tissue lipolysis in hormone-independent way through ANPTL4 up-regulation.

Modulation of hepatic lipid homeostasis by PPARδ agonists

As demonstrated in this thesis, PPARδ activity leads to triglyceride accumulation in the liver of non-transgenic and especially in mice over-expressing hPPARδ. This phenomenon is transient, at least in wild-type animals, but the question remains if this pharmacologically-induced temporary liver steatosis is still toxicologically relevant phenomenon. There is still much to be known about the progression from “simple” liver steatosis to non-alcoholic steatohepatitis (NASH) or from NASH to cirrhosis. These steps are crucial. 25-75% type 2 diabetic patients develop fatty liver phenotype [190]. Influx or synthesis of additional fat may trigger or speed up the progression to more advanced disease level in patients with metabolic syndrome or type 2 diabetes [38].

Although earlier reports indicate that PPARδ can be down-regulated in rodent liver after fasting [173], this nuclear receptor activity is responsible for fasting and exercise-induced metabolic transcriptional changes, which lead to major alteration in glucose and lipid metabolism and the liver is key organ in this process [31,142,191,192]. Therefore systemic delivery of potent PPARδ ligands will have profound influence on hepatic physiology either direct or indirect [10]. Stannard et al showed that 72 hours fasting increases intramyocellular lipid content in non-diabetic, physically fit men [193] and Moller et al demonstrated that intrahepatic lipid content in human subjects measured by 1H-magnetic resonance, increased significantly after the 36 h fasting period by 156% [51]. Rodent fasting physiology is also comparable with human. For example, 12–18h fasting in voles results in livers displaying steatosis with characteristic accumulation of triacylglycerols, while fatty acids prevalent in membrane

phospholipids decreases in proportion [194]. Additionally van Ginneken reports that 24h starvation in mouse leads to significant rise in hepatic triacylglycerols by 456% [195]. And other reports shows similar phenomenon [19,196]. Another metabolic area where PPARδ is particularly important is physical activity. PPARδ agonists were even proposed as exercise mimetics [121]. In work done by Hu et al, the authors demonstrated that after one bout of exercise in mice hepatic triglyceride species were found to be more abundant in the recovery phase, while phosphatidylcholines species were decreased [197]. The same authors also show that muscle triglyceride content was decreased during exercise presumably due to increased muscle fatty acid oxidation. This is in accordance to our findings where we were able to show promotion of pro-lipid oxidative transcriptional changes in muscle of non-transgenic and especially hPPARδ animals treated with GW501516.

In natural physiological state, PPARδ is being activated upon increased energy demands, governing a number of pathways, which lead to utilization of fat as an energy substrate [191,198]. Pharmacological activation of PPARδ using highly selective, potent, but artificial ligands like GW501516, initiates whole metabolic sequences without the actual energy demand. In such case, adipose tissue releases fatty acids, which are not immediately required to power the body organs. In work published by Berge et al, the authors demonstrated that tetradecylthioacetic acid (TTA) a PPARα and PPARδ dual agonist helps to improve blood hyperlipidemia in rats. They formed the hypothesis that free fatty acids (FFA) will be drained from the plasma into the liver, and oxidized at the expense of triglyceride accumulation in the hepatocytes. And draining of FFA by the liver will relieve the fatty acid pressure on adipose and muscle tissue where

glucose uptake and oxidation is inhibited by fatty acids [10]. Similar conclusion were drawn by Liu et al where they have shown that adenovirus-mediated liver restricted PPARδ activation reduces fasting glucose levels in normal and high fat diet - fed mice. This effect was however accompanied by hepatic glycogen and lipid deposition [128]. On the other hand however, still reasonable evidence supports the hypothesis that PPARδ agonism is protective against hepatic steatosis. In recent work by Lee et al, rats treated with GW0742 from 26 to 36 weeks showed improvement in fatty infiltration of the liver [199]. And in the MCD-diet induced mouse model of NASH, treatment with GW501516 ameliorates hepatic steatosis and inflammation by improving lipid metabolism and inhibiting inflammation [129]. Our observation and other reports [124] indicate that time of treatment is the key factor. The mechanism by how this is achieved is a matter of availability of substrate to infiltrate the liver (abundance of WAT deposits being lipolysed by PPARδ pharmacological activation) and production and steady build up of particular inducer of hepatic fatty acid oxidation. Experimental evidence shown in this thesis suggest that despite availability of WAT deposits, fat is cleared from the liver, as was demonstrated in wild-type animals in chapter 3. Liu et al proposed that PPARδ agonism protective role against hepatic steatosis relies on generation of protective mono-unsaturated and lowering lipotoxic saturated fatty acid levels [128]. Our findings showing time-depended increase in PPARα endogenous ligand (POPC) fully confirm and strengthen this hypothesis.

Together these data support a beneficial role for PPARδ ligands in the improvements of diabetes, but care need to be taken when considering treatment of patients with defined NASH with PPARδ agonists.

PPARα-PPARδ interaction

The successful use of the fibrates class of drugs as an efficient treatment for dyslipidemia, was attributed to activation of PPARα receptor [99].

However, in one of the first reports of using PPARδ ligands in PPARα-null animals, which came from DeLuca et al work [200], the authors found that administration of PPARδ agonists to mice lacking PPARα induces peroxisome proliferation in the liver a phenomenon formerly solely credited to PPARα activity. It opened up the question of functionality overlaps and mutual interactions. The findings presented in chapters 6 and 7 from this thesis unravel the complex system of interaction between these two receptors in controlling transcription of multiple sets of genes. Alterations in gene expression in PPARα-KO mice treated with the PPARδ agonist are also reflected in lack of phenotypic changes traditionally attributed to PPARδ agonism like mobilization peripheral lipid stores and subsequent weight reduction. On the other hand, we observed that the ability of PPARδ agonists of raising HDL levels could be still evident in PPARα-KO animals. Previous [127] and more recent [184] findings shown that PPARδ agonists may be effective drugs to increase reverse cholesterol transport and we have now demonstrated that PPARα signalling is not necessary in this process.

One of the important question which remains to be answered is whether small molecule agonism or antagonism of PPARα could have the same consequences for PPARδ-agonism as genetic ablation of PPARα had, as shown in this thesis. As we demonstrated, absence of PPARα blocks efficiently some important PPARδ agonistic attributes in relation to its anti-obesity properties. Adhikary et al also shown that for transcription of certain set of

genes siRNA-mediated removal of PPARδ protein is equal to ligand binding to existing receptor [153]. We confirmed that removal of PPARα or PPARδ has always two way consequences of up and down-regulations of whole group of genes. De-repression mechanism by removal of the functional nuclear protein from the system was also evident, when levels of POPC in PPARδ-KO animals were found to be elevated, when compared to wild-type animals. PPARδ agonist treatment had similar effect (Fig. 6.13). If the fibrate-responsive genes at least in part, belong to the group where ligand binding and ablation of the receptor causes the same transcriptional event, simultaneous therapeutic use of fibrate class of drugs and specific potent PPARδ agonists could prove counter-productive in medical application of PPARδ ligands. On the other hand, research from use of pan activators of PPARs such as punicic (PUA), jacaric acid (JAA) catalpic (CAA), and eleostearic acids (ESA) shows that all have demonstrated some promising health effects by acting as dual or pan agonists of PPARs [86]. In addition we demonstrated that mutual cooperation between PPARα and PPARδ in clearance of hepatic fat relies on generation of POPC, and endogenous PPARα ligand. However, care needs to be taken as evidence is constantly growing that PPARs can have mutual antagonistic effects and interaction between three subtypes of PPARs is important for understanding biology of these transcription factors. For example, Gustafsson et al found that dominant negative form of PPARδ will have positive and pronounced effect on PPARα and PPARγ signalling [148] and one of the recent report from Kocalis et al shows that neuron-specific deletion of PPARδ causes up-regulation of PPARγ and target genes of both PPARα and PPARγ, as well as genes of fatty acid oxidation [151]. The complex interplay between the isoforms of PPARs is

illustrated by the regulation of expression of cyclooxygenase-2 (COX-2) in rat brain astrocytes. The authors discovered that thiazolidinediones increase COX-2 expression via a PPARγ-dependent increase of PPARδ receptors [201]. Furthermore, they found that positive influence of PPARγ and negative influence of PPARα on PPARδ transcriptional activity occurs via regulation of the expression level of PPARδ. Additionally, the PPARδ-activation resulted in an increase of PPARα expression level, thus forming a positive/negative feedback loop [201]. PPARδ signalling serves as a connection of PPARγ and PPARα-dependent signals to target genes. A crossroad between PPARs was suggested previously. Zuo et al found that linoleic acid activates PPARδ and subsequently inhibits PPARγ activity [202]. And Shi et al showed that even stable expression of PPARδ isoform is sufficient to repress the induction of PPARα target genes [203]. In this way, not only the absolute levels of any one PPAR isotype but also the ratio of the PPAR isotype levels will control the activity of each member of PPAR family. Summarizing, when considering therapeutic use of PPARδ agonists or antagonists one needs to bear in mind that PPARδ can be also considered as regulator and gatekeeper of other PPAR subtypes for which clinically drugs are in use and therefore possible antagonistic interaction is likely to occur, when co-used with prospective PPARδ potent modulators.

Concluding remarks and future directions

Although the biology of PPARδ has not been fully deciphered, here we show the evidence that liver specific over-expression of human PPARδ in mouse liver promotes hepatic steatosis conferred through extensive peripheral adipose

tissue lipolysis and consequently an influx of fatty acids into the liver. As a result, PPARδ emerges not only as a master controller of lipid oxidation genes, but also as a modulator of transmembrane transport and WAT lipolysis possibly through ANGPTL4 activity, one of the key PPARδ target genes. This effect however, requires PPARα signalling regardless the levels of ANGPTL4. The mechanism by which the absence of PPARα inhibits PPARδ-induced lipolysis of adipose tissue is worth exploring, taking into consideration the importance of this mechanism in anti-obesity related drug research. Findings in this thesis also provide evidence for tight interplay between members of PPAR family. Investigation of the exact role the PPARδ has in controlling the two other PPAR subtypes would be also significant, bearing in mind that thiazolidines as ligands for PPARγ and fibrates for PPARα have been both useful and troublesome. Using PPARδ as indirect modulator of PPARα and PPARγ when challenging pathological aspects of metabolic syndrome could be promising way of improving mode of actions of these drugs.

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